THE EFFECTS OF EARLY EXTIRPATION OF THE THYMUS IN ALBINO RATS.*

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INTRODUCTION.

Marked differences have been observed in the character and intensity of the changes that follow extirpation of the thymus. These differences may be attributed in part to variations in the conduct of the experiments, but in part they appear to depend upon the particular species of animal used. Most of the recent experimental work has been done upon dogs and rabbits. Fewer, and for the most part poorly controlled observations have been made upon other animals, —goats, guinea pigs, cats, chickens, and frogs,—but they have not led to conclusive results.

The effect of removal of the thymus gland in white rats has not previously been investigated. For this reason, and because it was expected that disturbances in the calcium metabolism resulting from the loss of the gland at an early age might be manifested by changes in the teeth, a systematic study has been made during the past year of the effects of thymectomy upon white rats. The experiments were interrupted last spring by the outbreak of an epidemic, due to infection by a bacillus of the enteritis group, which destroyed many of the rats.[1] The work has been hampered by the sporadic occurrence of this infection, and to a lesser degree by the chronic lung disease which is so often found in rats. As the result of a year's work, therefore, we have only a limited number of uninfected rats from which conclusions as to growth and nutrition may be drawn. However, the experiments have been carefully controlled, and we feel justified in reporting the data, especially since the known nor-

* Received for publication, December 29, 1913.

[1] A study of the bacteriological and pathological findings in this disease will appear in a forthcoming number of the *Journal of Infectious Diseases.*

mal variations in growth and weight, as recently worked out in detail by Hatai (1) and Jackson (2), serve as further controls. Their figures emphasize the need for caution in drawing inferences from slight differences in weight. This is true even for animals of the same litter, maintained under apparently identical conditions.

The literature relating to the experimental extirpation of the thymus has been collected in the exhaustive reviews of Hammar (3), Wiesel (4), Klose (5), Biedl (6), Matti (7), and Hart (8). Since the effect of removing the thymus of rats has not previously been studied, reference will be made only to the observations that bear upon our work.

METHODS AND TECHNIQUE.

The removal of the thymus was performed upon young rats, ranging in weight from ten to twenty-five grams. When possible the age was accurately determined, but in some litters the age had to be estimated. Probably none of the operated animals were over twenty-one days old when the gland was removed. Two or more rats from each litter were reserved as controls, and kept in the same cage as the operated rats.

The operation was performed aseptically under ether anesthesia. A median incision was made in the lower part of the neck and continued over the upper half of the thorax. The sternum was then split as nearly as possible in the median line as far as the fourth or fifth intercostal space, and the edges were retracted. The triangularis sterni muscle was separated, exposing the thymus, which was carefully dislodged from below upwards with fine blunt forceps, and removed when possible, without laceration or separation of the two lobes. The friability of the gland made this the most difficult part of the operation. The sternum and skin were sutured with silk. Healing in almost all cases took place rapidly and without suppuration. It was found advantageous to use a simple positive pressure apparatus, to prevent collapse of the lung when the pleura was accidentally injured, and to institute artificial respiration when necessary.

In the earlier experiments the control rats were also subjected to etherization and the sternum was incised without removal of the

thymus. It was found later that the operation had practically no immediate effects, and this precaution was therefore considered unnecessary.

After the end of the nursing period, the rats were fed principally on bread, sunflower seed, and greens. Meat was not given, and the feeding of milk was given up, since it seemed to favor the spread of infection.

The operated rats together with the controls were kept under observation for varying periods up to six months, and were then killed. They were weighed weekly.

At autopsy the organs of the neck and thorax were removed together. After fixation the lungs and lower half of the heart were dissected off, and the rest of the tissue was embedded in paraffin and cut in series of 10 micra in thickness. In the earlier litters each section was mounted, but later only every tenth section was examined. When there was doubt as to the nature of the tissue, the intermediate sections were also studied.

The other organs were fixed in Zenker's fluid, and stained with hematoxylin and eosin. The bones and teeth were fixed in formalin and decalcified in phloroglucin nitric acid, or in some cases, in Müller's fluid. The adrenals were fixed in Müller-formol solution.

EXPERIMENTAL PART.

26 litters, comprising 118 rats, were used for this study. Of these, 82 were thymectomized, the remainder serving as controls. 19 rats died during or soon after the operation. 5 litters, including 14 operated rats and 11 controls, are still under observation. In 18 of the remaining 49 rats, extirpation was proved to have been complete by serial section through the entire neck organs. In one of the rats accessory thymus tissue was found. 5 of the 18 rats showed lesions characteristic of infection with the bacillus of the enteritis group mentioned above, and are excluded on this account.

The following abbreviated protocols, therefore, refer only to rats in which entirpation was proved to have been complete, in which no accessory tissue was present, and which were shown by microscopic study to be free from infection.

The data given in table I afford a decisive answer to the question

TABLE I.

Summary of Protocols.

Litter.	Sex.	Age at operation.	Weight at operation.	Weight at death.	Duration of life after operation.	Remarks.
A2	F.	14 dys.	14.1 gm.	58.5 gm.	31 dys.	Killed with chloroform.
A3	M.	14 dys.	16.3 gm.	66.0 gm.	31 dys.	Killed with chloroform.
A5	F.	14 dys.	14.6 gm.	62.0 gm.	31 dys.	Control: sternum incised.
A6	F.	14 dys.	14.6 gm.	65.0 gm.	31 dys.	Control.
B2	M.	15 dys.	16.1 gm.	86.9 gm.	43 dys.	Killed with chloroform.
B6	M.	15 dys.	16.4 gm.	98.6 gm.	43 dys.	Control: sternum incised.
E2	M.	10 dys.	12.0 gm.	77.5 gm.	58 dys.	Killed with chloroform.
E6	F.	10 dys.	12.0 gm.	59.0 gm.	58 dys.	Control.
F1	F.	10 dys.(?)	18.5 gm.	70.0 gm.	99 dys.	Killed with chloroform.
F4	F.	10 dys.	18.0 gm.	111.0 gm.	99 dys.	Control (infected).
M3	F.	13 dys.	11.0 gm.	33.0 gm.	70 dys.	Spleen removed 11 days after thymectomy.
M7	F.	13 dys.	11.0 gm.	49.0 gm.	82 dys.	Control: spleen removed at same time as M3.
O1	M.	20 dys.	21.0 gm.	107.0 gm.	120 dys.	Killed with chloroform.
O2	M.	20 dys.	21.5 gm.	106.0 gm.	120 dys.	Killed with chloroform.
O3	F.	20 dys.	23.0 gm.	87.0 gm.	131 dys.	Killed with chloroform.
O5	M.	20 dys.	23.5 gm.	122.5 gm.	120 dys.	Control.
O6	F.	20 dys.	21.5 gm.	83.0 gm.	131 dys.	Control.
P1	F.	23 dys.	24.5 gm.	137.0 gm.	130 dys.	Killed with chloroform.
P2	F.	23 dys.	24.5 gm.	111.0 gm.	130 dys.	Killed with chloroform.
P3	F.	23 dys.	24.5 gm.	118.0 gm.	130 dys.	Control.
S2	F.	14 dys.	17.0 gm.	63.0 gm.	95 dys.	Minute thymus rest (?).
S6	M.	14 dys.	18.0 gm.	72.0 gm.	90 dys.	Control.

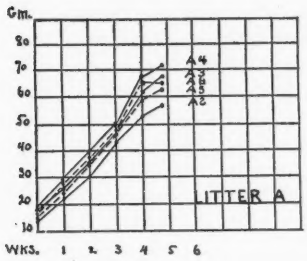
whether the thymus in rats is a vital organ. The period of survival after operation ranged from 45 to 131 days. The animals of the last group (litters O and P) showed no evidence of illness at that time. Among the litters at present under observation are two rats that have survived the operation for 170 days and are still in good health.

It is therefore proved that whatever influence the thymus may have upon growth and nutrition in the rat, it is not essential to life. Sexual maturity, according to Slonaker, is attained at ten weeks, although the maximum weight is not reached until after the first year. The period of observation is therefore sufficiently prolonged, in the case of litters E, M, F, S, O, and P, to carry the animals well past the onset of puberty, at which time in rats (Jackson) and in

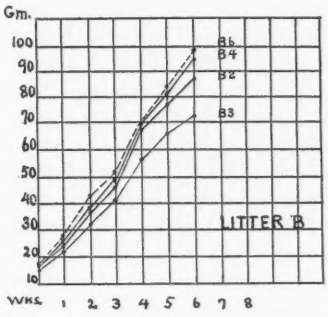
rabbits (Söderlund and Backman (9)), the thymus has begun its normal involution, and is probably no longer at the height of its functional activity. It is not reasonable to suppose that the fatal effects of thymus deprivation would appear only after the anatomic regression of the organ was well under way.

INFLUENCE OF THYMECTOMY UPON GROWTH AND NUTRITION.

An analysis of the weight curves (text-figures 1 to 8)[2] does not indicate a definite influence of thymectomy upon growth.

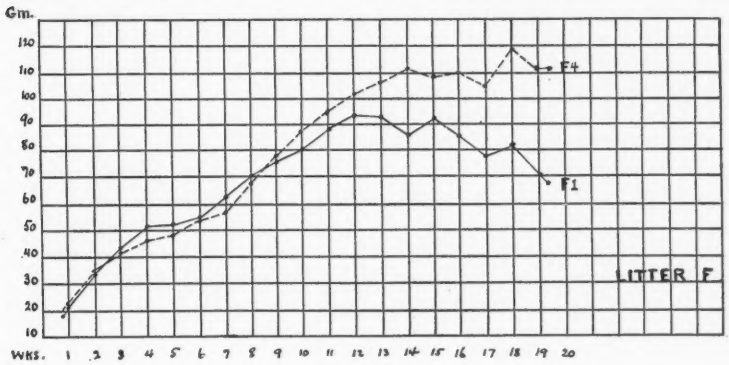


TEXT-FIG. 1.



TEXT-FIG. 2.

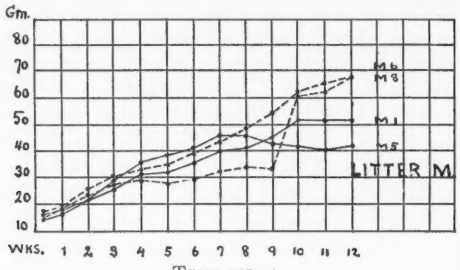
The ten completely thymectomized rats in which microscopic examination showed no evidence of infection were A2, A3, B2, E2, F1, O1, O2, O3, P1, and P2. Two of the rats, A2 and B2, showed



TEXT-FIG. 3.

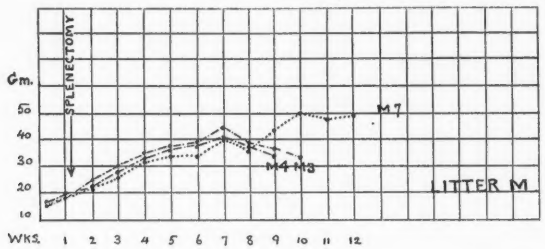
[2] In the charts the unbroken lines represent operated animals, the broken lines controls.

a slight retardation of growth as compared with healthy controls of the same litter. The differences in weight at the time when the



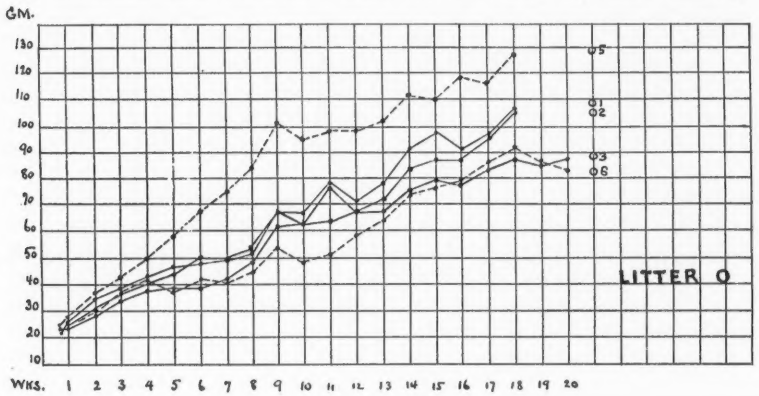
TEXT-FIG. 4.

animals were killed were insignificant and fall easily within the range of normal litter variation as determined by Jackson. Rat



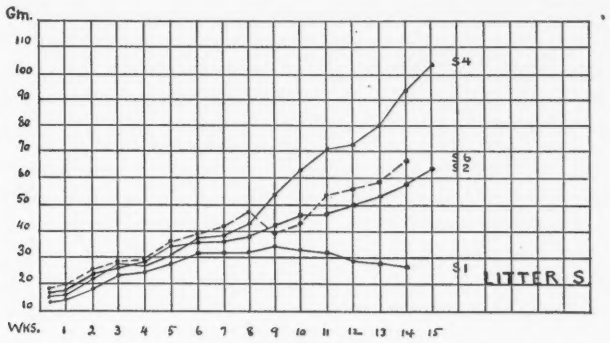
TEXT-FIG. 5.

A3 exceeded both controls. Rats O1, O2, and O3 were midway



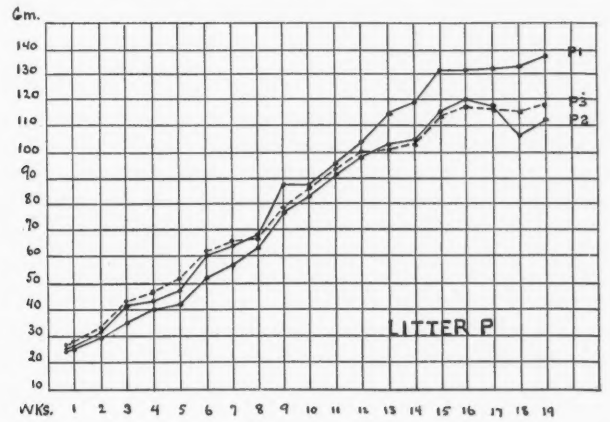
TEXT-FIG. 6.

between the two controls in weight. Rat P1 was above, Rat P2 below the control. Rat F1, ninety-nine days after thymectomy,



TEXT-FIG. 7.

weighed forty grams less than the normal control, F4, but even extreme variations such as this are apparently not uncommon in rats raised under laboratory conditions.[3]



TEXT-FIG. 8.

On the basis of these facts, and taking into consideration our data relating to rats in which small lobules of thymic tissue were

[3] Jackson, C. M., *Am. Jour. Anat.*, 1913, xv, 1. Compare table I, litter M7, in which the weights ranged at 44 days from 23.5 to 71.8 gm. The variations may of course be due to unrecognized pathological conditions.

found, but in which there must have been a marked functional deficiency, one must conclude that removal of the thymus in rats does not noticeably affect growth and nutrition.

BEHAVIOR OF THE OPERATED RATS.

If we leave out of consideration all symptoms that could be attributed to accidental infection and that could be duplicated in normal rats experimentally infected with *Bacillus enteritidis,* no peculiarities in behavior were noted. Particularly to be emphasized is the absence of nervous irritability, tremors, convulsions, and stupor. There was not the slightest evidence of mental deterioration in any of the operated animals, nor was there anything peculiar in their appearance or posture.

LOCAL CHANGES.

The large space left by the removal of the gland becomes replaced by adipose tissue, which for a long time, preserves the character of embryonic fat lobules.

The regional lymphatic glands in the operated rats show a marked hyperplasia. This is accompanied frequently by a distension of the lymph sinuses, with exfoliation of endothelial cells. In some rats the sinuses were found filled with red blood cells, many enclosed in phagocytes.

As regards the behavior of small fragments of thymus tissue not removed at operation, only a few general statements can be made, as a study of the minute regenerative changes do not fall within the scope of the work. The general capacity of small fragments of thymic tissue to regenerate did not appear to be so marked as the statements made by Klose and others led me to believe. The thymic rests found in serial sections were often extremely minute and showed no evidence of active proliferation. They were composed of densely packed small cells, among which were a few complexes of larger, paler epithelial cells. Fully formed, concentrically disposed Hassall bodies were only exceptionally seen. The identification of small masses of tissue as thymus was therefore somewhat difficult, and rested upon the absence of peripheral lymph sinus and the presence of the epithelial cell complexes. Because of the closer crowding of the cells, the staining of the thymus tissue was somewhat deeper than that of the adjacent lymph nodes.

A further differential point observed was the absence of necrotic lesions in the thymic tissue in rats infected with *Bacillus enteritidis*. The surrounding lymphoid tissue in these rats regularly contained areas of focal necrosis, but in not a single instance was the thymus or the rest of thymic tissue similarly affected. This is in accord with the well known insusceptibility of the thymus to tuberculosis and other specific infections.

THE INFLUENCE OF THYMECTOMY UPON OTHER ORGANS.

Skeletal System.—Particular attention was given to possible alterations in the bones and teeth. The femur, tibia, upper and lower incisors, and in some cases, the ribs, were examined. The methods used included partial decalcification in Müller's fluid, and staining with hematoxylin and carmine after Schmorl.

I have failed to observe any characteristic qualitative changes which might be brought into relation with the operation. Whenever marked retardation of growth occurred, the bones were shorter, the epiphyseal cartilage narrower, the cortical and trabeculæ more slender, and the marrow spaces larger than in the controls. In not a single instance, however, was there marked gross deformity, nor was there any irregularity in the processes of ossification. Rachitis-like lesions like those described by Klose (10) and Matti (11) in dogs, and by Soli (12) in rabbits were wanting.

The teeth also showed no irregularities in the calcification of the dentine, and none of the animals that were observed over a long period showed spontaneous infarctions such as have been described by Erdheim (13) and Toyofuku (14) in parathyroidectomized rats.

In the absence of chemical studies it seems unwarranted to deny the influence of the thymus in rats upon skeletal and dental calcification. The foregoing observations, however, certainly afford no histological evidence pointing to such an influence.

Adrenals.—There has been brought forward considerable pathological and experimental evidence indicating a correlation or antagonism between the thymus and the adrenals. Matti (11) states that in thymectomized dogs, there was regularly observed, on macroscopic examination, a hypertrophy of the adrenal medulla. Histologically, there were found the changes described by Stoerk

and von Haberer (15), as characteristic of hypertrophic medullary tissue. Other references to the condition of the adrenals after thymectomy are less definite. Soli found the adrenals diminished in weight in six chickens and increased in five; in eight guinea pigs it was increased in six and diminished in two. Nordmann (16) describes a hyperemia. Lucien and Parisot (17) found a slight diminution in the absolute weight, and no appreciable change in the relative weight of the adrenals after thymectomy.

Hyperplasia of the thymus after adrenalectomy is described by Calogero (18), Bonnet (19), and Auld (20), and, as pointed out by Hedinger (21), Hart (22), Wiesel (23), Pappenheimer (24), and others, occurs frequently in Addison's disease.

I have not found definite alterations in the adrenals in thymectomized rats, either in weight or in histological structure. The chromaffin staining tissue was invariably abundant, but not more so than in the controls. Changes in the chromaffin cells which could be interpreted as indicating a hypertrophy were not found. The cortex also showed no structural difference from that of the control ad-

TABLE II.

Weight of Adrenal Glands.

Rat.	Sex.	Operation.	Absolute weight.	Relative weight.
M1	F.	Thymectomy (I)[4]	0.030 gm.	1–1733
M5	F.	Thymectomy (C)[4]	0.020 gm.	1–2100
M6	M.	Control	0.020 gm.	1–3350
M7	F.	Splenectomy	0.020 gm.	1–2450
M8	M.	Control	0.011 gm.	1–6909
O1	M.	Thymectomy (C)	0.022 gm.	1–4818
O2	M.	Thymectomy (C)	0.024 gm.	1–4396
O3	F.	Thymectomy (C)	0.017 gm.	1–5118
O4	M.	Control	0.024 gm.	1–5083
O5	F.	Control	0.021 gm.	1–3952
P1	F.	Thymectomy (C)	0.030 gm.	1–4560
P2	F.	Thymectomy (C)	0.025 gm.	1–4432
P3	F.	Control	0.028 gm.	1–4214
S1	F.	Thymectomy (C)	0.013 gm.	1–2083
S2	F.	Thymectomy (C?)	0.021 gm.	1–3000
S4	M.	Thymectomy (I)	0.027 gm.	1–3233
S5	F.	Splenectomy	0.015 gm.	1–3233
S6	M.	Control	0.020 gm.	1–3600
U4	M.	Thymectomy (I)	0.020 gm.	1–1800
U7	M.	Control	0.017 gm.	1–2200

[4] In the tables, I = incomplete, and C = complete.

renals, although special staining of the lipoids was not attempted. The absolute and relative weights of the adrenals in some of the operated litters are appended.

Although at first sight the figures appear to indicate a relative increase in the weight of adrenals in the thymectomized rats, this is probably due to the relatively small weight of many of the operated animals rather than to an hypertrophy. That it cannot be directly attributed to the loss of thymus is indicated by the fact that the weight is relatively high also in the splenectomized rats, M7 and S5. The highest relative weights, moreover, are found in rats M1 and U4, which were infected and cachectic, and in which the extirpation of the thymus was incomplete.

Testes.—The condition of the testes in the operated animals and in the corresponding controls is given in table III.

TABLE III.

Testes.

Rat.	Age.	Operation.	Weight of testicles.	Size of testicles.	Spermatogenesis.
A3	45 dys.	Thymectomy		4.5 mm.	No spermatogenesis.
A4	45 dys.	Thymectomy (I)		12.0 mm.	No spermatogenesis.
A6	45 dys.	Control		6.5 mm.	No spermatogenesis. More mitotic figures than A3 and A4.
B2	58 dys.	Thymectomy (C)	1.51 gm.		Spermatogenesis.
B4	58 dys.	Thymectomy (I)	1.45 gm.		Spermatogenesis.
B6	58 dys.	Control	1.50 gm.		Spermatogenesis.
E2	68 dys.	Thymectomy (C)		16.0 mm.	Active spermatogenesis.
E3	68 dys.	Thymectomy (I)		11.0 mm.	No spermatogenesis.
O1	140 dys.	Thymectomy (C)	1.456 gm.		Active spermatogenesis.
O2	140 dys.	Thymectomy (C)	1.425 gm.		Active spermatogenesis.
O5	140 dys.	Control	1.664 gm.		Active spermatogenesis.
S4	104 dys.	Thymectomy (I)	1.130 gm.		Fairly active spermatogenesis.
S6	104 dys.	Control	0.605 gm.		Fairly active spermatogenesis.
U4	79 dys.	Thymectomy (I)	0.180 gm.		No spermatogenesis.
U7	79 dys.	Control	0.296 gm.		No spermatogenesis.

In litter A spermatogenesis was not fully established at forty-five days, when the rats were killed. In litter B, at fifty-eight days, both thymectomized and control rats showed spermatogenesis; and this

was true in all the other testes examined, except in litter E. Here the development of the testis was further advanced in E2 than in another male of the same litter, E3, in which the removal of the thymus was incomplete. The data are not sufficiently numerous to warrant a final conclusion, but as far as they go, they do not indicate a decided effect of thymectomy upon the testes.

The literature bearing on this question contains contradictory statements. The most extensive experiments made to determine the influence of thymectomy upon the development of the testes are those of Paton (25). In guinea pigs removal of the thymus was followed by increase in the average weight of the testes, amounting in one series to 46 per cent. Microscopic examinations were not made.

In chickens, rabbits, and guinea pigs Soli (26) obtained opposite results. The testicles in the thymectomized animals killed after five months, were smaller, weighed less than the controls, and failed to show active spermatogenesis. Lucien and Parisot also found a transient retardation in the development of the testicles.

Klose and Vogt (10) substantiated in dogs the findings of Paton in that they observed a transient hyperplasia of the testes, which disappeared in the cachectic stage. Matti (11), on the other hand, was unable to determine any relation between thymectomy and the onset of spermatogenesis.

In view of these conflicting statements and our negative findings in rats, it seems premature to attribute to the thymus a direct influence upon the male genital glands. That there exists an inverse relation, however, by which the involution of the thymus is retarded after early castration, may be considered proved, and the numerous experiments of Henderson (27), Goodall (28), Calzolari (29), Ranzi and Tandler (30), and Soli and Gellin (31) are in agreement on this subject.

Thyroid.—In many of the operated animals the thyroid showed a slight variation in structure from that of the controls. The acini were smaller, the epithelium higher, and the colloid less abundant and less densely stained than in the controls. These differences, however, were not striking, and marked epithelial hyperplasia, with papillary infolding of the epithelium, such as Matti (11) describes

in thymectomized dogs, was not seen. The impression gained was that the alterations in the thyroid were not specific, but merely accompanied the arrested development. They were equally manifest in rats whose development was retarded by infections or other causes.

Spleen.—Soli found the weight of the spleen in chickens and rabbits unaffected by thymectomy. Lucien and Parisot also found no changes in the weight, but observed in certain cases an increase in the number and size of the follicles. Perrier (32), Klose and Vogt, and Matti have also observed a follicular hyperplasia, followed by atrophy in the athreptic stage.

Klose (5) concludes from these facts that the spleen "may to a certain extent and after a certain interval, take over the function of the ablated thymus." Late extirpation of the thymus, which ordinarily causes only transitory "rachitis-like disturbances of growth," if combined with splenectomy, leads to death within a few weeks. Matti (7) disputes both the facts and the inferences that Klose draws.

I have repeated this experiment in three rats, M3, M4, and S3. In M3 and M4, the spleen was extirpated 11 days after thymectomy. The animals gained steadily in weight until the 47th day after thymectomy, when the weight curve began to decline. M4 was found dead and partially eaten on the 66th day after operation. M3 became emaciated and weak, and developed diarrhea. It was killed on the 70th day, and extirpation was proved by serial section to have been complete. A control rat, M7, in which the spleen alone had been removed, weighed at this time seventeen grams more than M3, but also showed a retardation of growth as compared with the unoperated controls, M6 and M8.

This experiment proves that a rat may live for at least fifty-nine days after extirpation of both thymus and spleen. Since the microscopic examination showed ulcerative lesions in the intestine and inflammatory foci in the lungs, it is probable that death was due to intercurrent infection rather than to the lack of these organs.

In rat S3, which was splenectomized fifteen days after removal of the thymus, there was a normal gain in weight surpassing that of a control rat of the same litter (S5) in which the spleen alone had

been extirpated, but lagging somewhat behind the two unoperated controls. It was found dead and partially eaten eighty-eight days after thymectomy. Since the neck organs could not be examined serially, it was not possible to be sure that the thymus had been completely removed.

The absolute and relative weights of the spleen in our series of animals are given in table IV. There is wide variation in the weights

TABLE IV.

Weight of Spleens.

Operated rats.		Controls.	
A2	0.25 gm.		
B2	0.62 gm.	B6	0.325 gm.
B3	0.22 gm.		
M1	0.19 gm.	M6	0.205 gm.
M5	0.19 gm.	M8	0.285 gm.
O1	0.397 gm.		
O2	0.301 gm.	O5	0.396 gm.
O3	0.270 gm.	O6	0.238 gm.
P1	0.357 gm.		
P2	0.271 gm.	P3	0.292 gm.
S1	0.192 gm.		
S2	0.425 gm.		
S3	0.818 gm.	S6	0.560 gm.
U4	0.220 gm.	U7	0.166 gm.

and those of the thymectomized rats are sometimes heavier and sometimes lighter than those of the controls. The same variation was noted in the histological pictures. The size of the follicles in well nourished rats was large; in athreptic animals an atrophy of the lymphoid tissue occurred regularly. In no case where the nutrition of operated and control rats was equally good, were striking differences found in the histological picture. It seems therefore that thymectomy in rats does not lead to constant or definite alterations in the structure of the spleen.

Blood.—The differential blood counts[5] on four operated litters are given in table V. The counts are not sufficiently numerous to

[5] I wish to express my gratitude to Miss Gertrude Fisher for making the blood counts. Although the counts were not continued for a longer period than five weeks after the operation, the data are given since they may prove of use to other workers in this field.

TABLE V.

Litter M. Differential Count Made on August 14, 1913, Five and One Half Weeks after Thymectomy.

		Polymorphonuclears.	Small lymphocytes.	Large lymphocytes.	Large mononuclears.	Transitionals.	Eosinophils.
M1	Incomplete thymectomy; infection	36	26	22	8	8	0
M2	Complete thymectomy and splenectomy; no infection	46	18	22	10	4	0
M3	Complete thymectomy and splenectomy; no infection	21	38	25	8	8	0
M4	Complete thymectomy	28	29	20	11	12	0
M5	Control	30	16	43	7	4	0
M6	Control: splenectomy	30	27	30	13	5	0
M7	Control	35	19	24	17	5	0

Litter S. Operated July 22, 1913.

S1	Complete thymectomy						
	July 24	17	56	22	12	3	0
	July 31	41	16	17	15	11	0
	Aug. 9	41	31	16	11	1	0
	Aug. 14	34	32	24	3	7	0
	Aug. 21	36	26	25	8	5	0
S2	Complete(?) thymectomy						
	July 24	23	26	30	7	4	0
	July 31	39	29	22	5	5	0
	Aug. 9	35	18	31	13	3	0
	Aug. 14	30	21	27	14	8	0
	Aug. 21	31	15	32	14	8	0
S3	Thymectomy, splenectomy (Aug. 6)						
	July 24	17	45	24	9	4	0
	July 31	42	20	26	8	4	0
	Aug. 9	33	27	28	4	8	0
	Aug. 14	46	34	9	6	5	0
	Aug. 21	43	8	17	18	14	0
S4	Control: splenectomy						
	July 24	13	60	20	4	3	0
	July 31	11	76	8	2	3	0
	Aug. 9	15	61	17	4	3	0
	Aug. 14	44	18	22	11	5	0
	Aug. 21	22	46	21	7	4	0

TABLE V.—*Concluded.*

		Polymorphonuclears.	Small lymphocytes.	Large lymphocytes.	Large mononuclears.	Transitionals.	Eosinophils
S5	Control: no infection						
	July 24.....	14	68	13	1	4	0
	July 31.....	8	58	30	1	3	0
	Aug. 9.....	16	53	18	6	2	0
	Aug. 14.....	15	46	20	12	7	0
	Aug. 21.....	25	25	35	7	8	0
	Litter W.[6] *Operated November 22, 1913. Still under Observation.*						
W1	Thymectomy						
	Oct. 27.....	46	14	14	26[7]		0
	Nov. 6.....	28	39	22	11		0
	Nov. 12.....	42	24	26	8		0
	Nov. 17.....	40	32	20	8		0
	Nov. 25.....	50	25	29	6		0
W2	Thymectomy						
	Oct. 27.....	33	48	10	9		0
	Nov. 9.....	35	54	3	8		0
	Nov. 12.....	43	27	31	9		0
	Nov. 17.....	48	14	27	9		2
	Nov. 25.....	32	15	46	7		0
W3	Thymectomy						
	Oct. 27.....	60	18	14	8		0
	Nov. 1.....	58	20	16	6		0
	Nov. 12.....	43	21	31	5		0
	Nov. 17.....	49	15	31	5		0
	Nov. 25.....	49	10	34	8		0
W4	Control	22	62	9	7		0
		13	46	36	5		0
		28	35	14	14		0
		37	20	29	14		0
		44	32	18	6		0
W5	Control	13	52	26	9		0
		11	63	19	7		0
		44	25	24	7		0
		23	40	33	4		0
		40	25	27	4		4

warrant a minute analysis or to permit of generalizations. There is an evident tendency, particularly in litter S, towards the occurrence of a relatively high proportion of polymorphonuclears during the first weeks after operation. Since the wounds healed aseptically

[6] The total leucocyte count in this litter showed no differences between operated rats and controls. Leucocyte counts were also made on the rats of litters S and P, and no variation from the normal was found.

[7] Large mononuclears and transitionals were counted together.

and the polynucleosis persisted after complete healing, the change in the blood picture is probably due to the loss of the thymus rather than to the effect of the operation.

Previous studies of the blood changes in other animals following thymectomy are contradictory. Seiler (33), who made blood counts upon one of Matti's operated litters of dogs, found a delay in the establishment of the normal percentage of polynuclears; the lymphocytic count remained high for a longer period than in the controls. Matti attributes this to the general retardation in development. However, an inspection of the actual counts made by Seiler several months after the operation shows that the percentage of polymorphonuclears in the thymectomized dogs was slightly higher (72 to 81 per cent.) than in the two controls (65 to 68 per cent.).

Lampé and Liesegang (34), who carried out blood studies on Vogt's animals, found a progressive diminution of the lymphocytes to far below their normal values. This is more in accord with my findings, and is also in harmony with the well known occurrence of relative lymphocytosis in Status lymphaticus, Basedow's disease, and other alterations of the ductless glands commonly associated with hyperplasia of the thymus.

CONCLUSIONS.

It has been shown that white rats may survive complete extirpation of the thymus for at least 131 days, even when the operation is performed within the first two weeks of life.

Removal of the thymus does not produce an arrest or retardation of body growth and development.

Qualitative changes in the skeletal system or teeth have not been found. In emaciated, weak animals osteogenesis is less active than in healthy rats, and the long bones are smaller and more delicate in structure. Such quantitative differences appear to depend upon the general nutrition, are equally pronounced in rats whose development is retarded from other causes, and cannot be referred specifically to loss of thymus function.

No constant or characteristic alterations were detected in the spleen, testes, adrenals, or thyroid. Whatever functional correla-

tions may exist between thymus and any or all of these organs are not evident from the occurrence of histological changes after the removal of the thymus.

The relative proportion of lymphocytes in the blood is diminished for the first few weeks after the operation. We have not determined how long this alteration in the leucocytic formula persists.

Since this paper went to press an article has appeared by Klose,[3] describing briefly the results of thymus extirpation in pigs, goats, rats, and chickens. In rats thymectomized on the fourteenth day, there followed a progressive cachexia terminating in death after eight to ten weeks. Disturbances in ossification, which macroscopically and microscopically were identical with those of human rickets, developed also in the ribs and long bones. Some of Klose's litters failed to show these lesions, and this negative result is explained as having been due to the presence of thymic tissue within the thyroid gland.

The observations of Klose are in direct contradiction to the negative results described in this paper. Since the possible presence of accessory thymus tissue either within the thyroid or elsewhere was carefully excluded in my experiments, the discrepancy between my findings and those of Klose cannot be explained upon this basis.

Since this paper was sent to the publisher I have studied two additional rats which were killed 185 days after operation. Minute examination of a complete series of the neck organs, including the thyroid, failed to show any tissue which could be interpreted as thymus. The bones showed no rachitic changes.

The infective origin of rachitic and osteomalacic lesions in rats has been established by Morpurgo. Although reference is made by Klose to Morpurgo's work, the data given by him do not enable one to judge whether this infection was definitely excluded.

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THE RELATION OF THE GASTRO-INTESTINAL TRACT AND CONTENTS TO THE BODY-WEIGHT IN RABBITS.*

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In some experimental work on rabbits in this laboratory, in which the results were based on the body-weight, it has been found necessary to avoid the error introduced by the variation in the weight of intestinal, stomach, and bladder contents. In view of a possible wider interest in the data collected for our use, we report them in this paper.

Joseph[1] examined one hundred rabbits for the purpose of determining the proportion of body-weight which should be allowed for stomach and cecum contents when the dosage of various drugs is based on the weight of the animal. He gives figures for stomach and cecum contents of males and females, and for the stomach and cecum minus contents. The results given in this paper deal collectively with the contents of the stomach, small intestine, cecum, large intestine, and rectum, of both sexes; the figures also include the weights of the organs after the contents had been removed, in normal, castrated, and thyroidectomized animals. The data in the literature upon this subject are not sufficiently complete to meet all conditions nor to justify the formation of a rule which will cover all cases; hence it became necessary to determine the exact weights in each animal used. The error in body weight due to bladder contents may be obviated by applying digital pressure on the abdomen in the region of the bladder as soon as the animal is dead and the muscles are relaxed. This method was used in every case, and throughout the paper the weight after removal of urine is spoken of as gross body-weight. The problem of eliminating the error

* Received for publication, January 8, 1914.

[1] Joseph, D. R., *Jour. Exper. Med.*, 1909, xi, 36.

introduced by inclusion of the stomach and intestinal contents is more difficult. The animal was weighed before and after the urine was expelled, and then the whole tract from the cardiac end of the stomach to the lower end of the rectum was removed, with as little of the mesentery as possible. The tract was then weighed with its contents. The removal of the latter was accomplished by first cutting the tract at the junction of its several parts; then the stomach was opened along its greater curvature and the material within was scraped away without the removal of any of the mucous membrane. The remaining parts were placed on a smooth board and by means of a small piece of sheet cork, which does not cut the tissue, the contents were forced out by pressure along the outside. This was continued until all the material was removed. The stomach and intestines, now minus their contents, were weighed collectively, and their weight was subtracted from that obtained before the removal of the contents, thus giving the content weight which when subtracted from the gross body-weight gave what we shall call the reduced body-weight. The method of clearing the intestines of their contents by allowing a current of water to pass through introduces a considerable error by the water that remains in the folds of the lining mucous membrane, while with the present method a comparatively small error is made. The animals were killed by coal gas when it happened to be most convenient, without respect to feeding time, and this undoubtedly accounts to some extent for the wide variation in the weight of gastro-intestinal contents.

Among the animals examined there were thirty-one castrated and thirty-three normal males, twenty-one spayed and thirty-one normal females, five thyroidectomized males, and six thyroidectomized females. In the tables the averages are arranged in ascending order with respect to the reduced body-weight. In column 1 of the first four tables the numbers indicate the limits between which the reduced body-weight varies.

In tables I to IV it will be observed that the absolute weights vary directly with the reduced body-weights, while the weights per kilo, as seen in columns 8 and 9, vary inversely with the reduced body-weight. In table V the averages of the twenty-one spayed females are compared with those of the thirty-one controls. It will be noted

that the reduced body-weight is slightly more in the spayed than in the normal animals and that the absolute weights vary even with this slight difference. In table VI where the averages of the thirty-three normal and thirty-one castrated males are compared, the absolute weights vary with the reduced body-weight without exception. In table VII where the averages of the whole group of sixty-four castrated and control males are compared with those of the whole group of fifty-two spayed and control females, it will be noted that the average of the gastro-intestinal tract plus the contents is slightly more in the males that have a lower average for reduced body-weight. This is due to the contents, as shown by column 6. The weight of the tract alone varies with the reduced body-weight.

In table VIII is given a comparison of the averages of the sixty-four normal animals, both males and females, with fifty-two operated males and females, and here again there is no exception to the rule that the variation in reduced body-weight, although small, is accompanied by a direct variation of the gross body-weight, weight of gastro-intestinal tract plus its contents, the same tract minus its contents, and the contents alone.

The same is true without exception in table IX, where the thyroidectomized males and females are compared.

In tables V to IX there is a constant apparent contradiction to tables I, II, III, and IV, in that the weights per kilo as shown in columns 8 and 9 vary directly with the reduced body-weight. The difference between the averages for the reduced body-weight is so small in tables V to IX that one would scarcely expect the same rule to hold without exception, and this is noted with few exceptions to be true when a comparison is made between groups with a wider variation in reduced body-weight (tables I to IV).

The variation between males and females (table VII) or between operated and control animals of either sex (tables V and VI) in cases of castration (table VIII) or thyroidectomy (table IX) is not sufficiently marked, it seems to me, to justify the conclusion that there is a constant difference due to sex or the experimental conditions here mentioned, in as far as the organs in question are concerned.

Table X, in which are included the averages for the eleven thyroid-

ectomized and the sixty-four normal animals, shows a marked difference in that the absolute weights and the weights per kilo of the thyroidectomized group are much less than those of the normal group, especially when we notice that the reduced body-weight was considerably greater in the thyroidectomized animals. This is not due to thyroidectomy because the normal animals used as controls in the same experiment correspond closely. This variation between the two series seems too large to be explained on the basis of a normal

TABLE I.

Spayed Females.

Reduced body-weight, in kilos.	No. of animals.	Gross body-weight, in kilos.	Weight of gastro-intestinal tract plus contents, in gm.	Weight of gastro-intestinal tract, in gm.	Weight of gastro-intestinal contents, in gm.	Reduced body-weight, in kilos.	Weight of gastro-intestinal tract, in gm. per kilo of reduced body-weight.	Weight of gastro-intestinal contents, in gm. per kilo of reduced body-weight.
0.787–1.684	6	1.529	334.0	105.0	229.0	1.300	81.0	177.0
1.778–1.976	4	2.114	354.0	123.0	231.0	1.883	65.0	122.0
2.018–2.471	6	2.584	422.0	145.0	277.0	2.307	62.0	120.0
2.554–2.861	5	3.035	437.0	163.0	274.0	2.761	59.0	99.0

TABLE II.

Normal Females.

0.756–1.798	7	1.710	330.0	106.0	224.0	1.486	72.0	151.0
1.838–2.028	7	2.175	339.0	106.0	233.0	1.942	55.0	119.0
2.072–2.167	7	2.378	380.0	123.0	257.0	2.121	58.0	121.0
2.260–3.030	10	2.698	380.0	129.0	251.0	2.447	53.0	103.0

TABLE III.

Castrated Males.

1.142–1.572	6	1.651	327.0	100.0	227.0	1.424	71.0	160.0
1.614–1.876	6	2.087	377.0	114.0	263.0	1.824	63.0	144.0
1.916–2.195	10	2.346	399.0	128.0	271.0	2.075	62.0	131.0
2.210–3.338	9	2.873	452.0	161.0	291.0	2.582	62.0	113.0

TABLE IV.

Normal Males.

1.328–1.632	7	1.706	310.0	102.0	208.0	1.498	68.0	139.0
1.652–1.805	5	1.973	353.0	120.0	233.0	1.740	68.0	134.0
1.812–1.978	9	2.165	377.0	111.0	266.0	1.898	59.0	140.0
2.112–3.009	12	2.628	388.0	130.0	258.0	2.370	55.0	110.0

TABLE V.

Group.	No. of animals.	Gross body-weight, in kilos.	Weight of gastro-intestinal tract plus contents, in gm.	Weight of gastro-intestinal tract, in gm.	Weight of gastro-intestinal contents, in gm.	Reduced body-weight, in kilos.	Weight of tract per kilo of reduced body-weight.		Weight of contents per kilo of reduced body-weight.	
							In gm.	Per cent.	In gm.	Per cent.
Control females	31	2.284	364.0	122.0	242.0	2.042	60.0	6.0	119.0	11.9
Spayed females	21	2.300	387.0	134.0	253.0	2.048	65.0	6.5	123.0	12.3

TABLE VI.

Control males	33	2.207	363.0	117.0	246.0	1.961	60.0	6.0	125.0	12.5
Castrated males	31	2.315	396.0	129.0	267.0	2.048	63.0	6.3	130.0	13.0

TABLE VII.

Castrated and control males	64	2.259	379.0	123.0	256.0	2.003	61.0	6.1	128.0	12.8
Spayed and control females	52	2.291	373.0	127.0	246.0	2.045	62.0	6.2	126.0	12.6

TABLE VIII.

Control males and females	64	2.244	364.0	120.0	244.0	2.000	60.0	6.0	122.0	12.2
Operated males and females	52	2.309	392.0	131.0	261.0	2.048	64.0	6.4	128.0	12.8

TABLE IX.

Thyroidectomized males	5	2.098	270.0	90.0	180.0	1.918	47.0	4.7	95.0	9.5
Thyroidectomized females	6	2.424	338.0	108.0	230.0	2.194	50.0	5.0	105.0	10.5

TABLE X.

Normal males and females	64	2.244	364.0	120.0	244.0	2.000	60.0	6.0	122.0	12.2
Thyroidectomized males and females	11	2.276	307.0	100.0	207.0	2.069	49.0	4.9	102.0	10.2

variation. As an explanation, however, it appears reasonable that it may have been due to a difference in the food supplied. The thyroidectomized animals and their controls constituted a series which were killed in the spring after having been fed through the winter for the most part on grain, while the sixty-four normal animals with which they are compared in table X were killed in the fall after having been fed through the summer on grass and vegetables.

Joseph concludes that for practical purposes 10 per cent. of the body-weight of a rabbit should be allowed for the stomach and cecum contents. It will be noted that this percentage agrees with the corresponding percentages in this paper as seen in column 11, tables V to X, when it is kept in mind that in the present case the contents of the whole gastro-intestinal tract are included.

According to tables I to IV, which I believe is the more logical grouping, the smaller animals show a larger weight per kilo of the gastro-intestinal contents and gastro-intestinal tract than larger animals, which is also in accord with Joseph's results.

ON THE NATURE OF THE OPSONIC SUBSTANCES OF NORMAL SERA.*

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Plate 40.

In spite of the many investigations upon the phagocytosis-enhancing substances of normal and immune sera, there is still much difference of opinion regarding the nature of the antibodies by which these effects are brought about; and the lack of agreement has centered chiefly upon the question of whether the opsonins are separate antibodies, independent of those previously known, or whether opsonic effects can be attributed to the activities of the alexin and sensitizer, acting individually or in coöperation.

Investigations into the nature of the opsonins of normal sera have pointed, with increasing probability, toward the participation of alexin or complement in the opsonic effect. In their earliest communications Wright and Douglas (1) noted the heat sensitiveness of normal opsonins, and since then many other similarities between the opsonic and alexin constituents of the serum have been observed.

The experiments of Hektoen and Roediger (2) showed that, like complements, opsonins are inhibited ("neutralized or bound") by various salt solutions and by formalin.

The work of Levaditi and Inmann (3) brought out a number of points of similarity which may be tabulated as follows: (*a*) both alexin and opsonin may be unspecifically absorbed out of serum by normal bacteria; (*b*) both are absorbed by cellular detritus or organ emulsions; (*c*) neither is present normally in aqueous humor, but both appear proportionally, with equal rapidity, two or three hours after puncture of the eye; (*c*) in edematous fluids there is a quantitative parallelism between the two.

Muir and Martin (4) further showed that opsonin, as well as complement, is fixed by specific precipitates and sensitized red blood cells.

It seems fairly clear in consequence of these and other investigations that the substances which exert opsonic action and those which

* Received for publication, January 15, 1914.

activate sensitized complexes cannot be definitely separated from each other by any of our present methods. Nevertheless the prevailing opinion seems to be that the alexin is an important participant in normal opsonic action, but that the process itself is dependent upon a complex mechanism in which normal amboceptor or sensitizer coöperates with the alexin, just as this is supposed to take place in the bactericidal or occasional hemolytic effects of normal sera.

This is the opinion of Dean (5), who believes that the mechanism of normal and of immune opsonic action is, in principle, the same, but that in normal serum the amount of sensitizer is so small that the destruction of the alexin reduces opsonic activity to a degree which is too slight to be measurable. Neufeld (6) in a recent treatise expresses his opinion unqualifiedly as follows: "The phagocytic antibodies of normal and immune sera may be divided into two classes: first, the 'tropins,' which are simply constituted substances, do not require the coöperation of complement and, therefore, are still active after serum has been heated; and second, the complex 'opsonins,' in the case of which, just as in bacteriolysis and haemolysis, a coöperation of amboceptor and complement is involved."

We shall not discuss differences of opinion regarding immune opsonins or bacteriotropins. As far as the normal opsonins are concerned, the views of Dean and Neufeld are apparently shared by Levaditi and Inmann, Cowie and Chapin (7), and a number of other investigators, all of whom succeeded in showing that the opsonic power of normal serum destroyed by inactivation, may be restored, in part, by the addition of diluted normal serum, too slight in amount to produce powerful phagocytosis by itself.

An analysis of the opsonic properties of the alexin fractions produced by the methods of Ferrata, Sachs-Altmann, and others was therefore undertaken; but after the work was well under way it was found that a similar investigation, previously overlooked, had been carried out by Hata (8) in 1908.

Hata found that, as in the case of complement, opsonin is divided by dialysis into two parts, neither of which is active alone, but together in proper amounts, opsonic action can be restored. It is important to note, however, that the reconstructed opsonin was not, in his experiments, as strong as the original undivided substance. This, then, draws a complete parallel between alexin or complement and opsonin, as analyzed by the method of complement-splitting. Our results at this time, however, had already begun to show definite discrepancies from those obtained by Hata, and we continued and repeated experiments in order to rule out accidental causes and to arrive at a definite result.

We shall now describe certain general features of our technique, which were employed in all the experiments, in order that we may omit in the protocols all but the particular controls which have definite bearing on the individual experiment.

METHODS EMPLOYED IN ALEXIN-SPLITTING AND IN THE CONTROL OF HEMOLYTIC ACTIVITIES.

In the preliminary experiments rabbit serum was occasionally used. Fresh normal guinea pig serum was used throughout our later experiments and in the work reported in this paper. The plan of procedure was to separate the alexin or complement into its fractions by various methods, and then to test these split products for both alexin and for opsonic effects, alone and reunited, in parallel series.

We employed for the purpose of alexin-splitting the dialysis method of Ferrata, the dilution method of Sachs and Altmann (dilution with slightly acidified distilled water), and the passage of carbon dioxide through the diluted serum, as advised by Liefmann and others.

In each experiment we determined that each fraction alone was completely inactive in hemolyzing sensitized cells, and that when put together activity was restored. This was done both immediately after accomplished splitting, and again at the time at which the opsonic tests were set up, in order to control possible deterioration of the globulin fraction, which is known to proceed rapidly when this constituent is kept for a few hours dissolved in salt solution.

The error which is most difficult to avoid in the experiments consists in an incomplete separation of the two alexin fractions. We shall not at present go into the work of Bronfenbrenner and Noguchi (9) which throws doubt upon the actual existence of two separate alexin fractions. This will be considered in connection with later experiments. However this may be, it is unquestionably difficult to obtain either an albumen or a globulin fraction devoid of traces of hemolytic activity by any of these methods, and apparently pure fractions may often be shown to be hemolytically active if tested, in sufficient quantity, with highly sensitized cells.

Hence the sensitized red cells which were used for control (sheep cells and anti-sheep rabbit serum) were saturated with sensitizer by the addition of an excess of immune serum, and subsequently washed. In consequence they formed an extremely delicate indicator of hemolytic action, and in the control tests the alexin fractions were added in quantities which corresponded often to many multiples of the amounts powerfully active when the two elements were added together. With a control as rigid as this, we encountered many difficulties in completely splitting the complement. Both the carbon dioxide method and the Sachs-Altmann method of dilution usually yielded a globulin fraction which was inactive, though the albumen fraction in these cases was often slightly hemolytic. For this reason in many of our earlier experiments we combined the carbon dioxide globulin fraction with the dialysis albumen fraction, or end-piece. In later experiments we used the dialysis method entirely, as being likely to produce the least chemical alteration in reaction, etc.

In carrying out the dialysis method our final procedure was as follows: Amounts of 5 to 10 c.c. of fresh guinea pig serum were divided into two parts,

one part being preserved under the same conditions of light and temperature as the dialyzed part in order that it might be used as a normal complement control. The other half was diluted with two parts of distilled water and placed into a fish bladder dialyzing bag which was hung into a jar of distilled water. Into this jar a steady trickle of distilled water was allowed to drip out of a five gallon demijohn, the water being allowed to splash close to the dialyzing bag in order to establish currents; it was removed by siphon arrangement which took the water from the bottom of the jar. In this way a constant level was maintained, and from 5 to 10 gallons of distilled water were run through during the course of from 24 to 36 hours. After 24 hours small quantities were removed from the bag, centrifugalized and, after being rendered isotonic with 10 per cent. salt solution, were tested against sensitized cells. When the albumen fraction or end-piece, in amounts equivalent to about 0.3 c.c. of the original serum, had been found to be entirely inactive, the bulk of the serum was taken out of the bag and centrifugalized until the supernatant fluid was clear. This was pipetted away and rendered isotonic with 10 per cent. salt solution, constituting our albumen fraction (end-piece). The globulin fraction was dissolved in an amount of salt solution equivalent to the volume of the original serum it represented. It is noteworthy that the globulin fraction so prepared was never in any of our experiments entirely inactive if used in sufficient quantity with sensitized cells.[1] In the majority of cases a rapid hemolysis occurred even when this globulin fraction (mid-piece) was used in quantities equivalent to 0.1 c.c. of the original serum. For this reason it was always reprecipitated by adding distilled water and again dissolved in salt solution. One reprecipitation rarely sufficed, and in some cases there was slight hemolytic activity even after two or three reprecipitations. Although reprecipitation always involved a certain amount of loss it was continued in every case until the mid-piece, dissolved in salt solution, was found to be entirely inactive. The persistence of hemolytic properties in the dialyzed globulin fraction after precipitation is in itself an interesting phenomenon in that it indicates how slight may be the amount of albumen fraction (end-piece) still capable of hemolyzing in the presence of the globulin, before complete separation has been attained. It seems hardly credible that after two reprecipitations enough end-piece can be still mechanically adherent to the globulin to cause hemolysis, especially since after complete separation the amount of albumen fraction necessary for activation of mid-piece rarely amounts to less than the equivalent of from 0.025 to 0.05 of the original serum. While no definite explanation can be offered for this peculiarity, it seems to point to the fact that in the original serum the fractions are present as a complex and not separately.

Incidentally in carrying out the preliminary titrations we were able to confirm a number of the observations of Hecker (10) concerning the inactivation which results if the globulin and albumen fractions are allowed to stand together for any length of time. If the two were allowed to stand at room temperature for from two to five minutes before the cells were added, marked inhibition of hemolysis was the result. In some cases distinct inhibition was induced when

[1] This fact has also been repeatedly encountered by Mr. Maltaner, working in our laboratory.

the albumen portion was first run into the tubes containing the red cells and the globulin fraction immediately after it. If the order was reversed prompt laking resulted.

METHODS EMPLOYED IN THE PHAGOCYTIC ESTIMATIONS.

Throughout our experiments twenty-four hour cultures of *Staphylococcus pyogenes aureus* on slightly acid agar were used. Emulsions of this were made in salt solution.

The leucocytes employed were obtained from guinea pigs in the following way: when an experiment was to be set up during the morning, the guinea pigs were injected intraperitoneally with five to ten cubic centimeters of a thin aleuronat emulsion on the preceding evening. When the experiment was delayed until the afternoon a similar injection was made early in the morning. As soon as the alexin fractions had been found inactive alone, and active together in the hemolytic tests, the guinea pig was killed, a small incision made in the abdomen, and ten cubic centimeters or more of citrate solution were run into the peritoneum. By gentle agitation this was made to mix with the exudate in the peritoneal cavity and was taken out with nipple pipettes into centrifuge tubes. By gentle centrifugation the clumps were first removed, the supernatant fluid, consisting of an even leucocytic emulsion, was pipetted off, and the leucocytes were washed in two or three changes of salt solution by slow centrifugation to avoid clumping. They were finally emulsified in salt solution and kept in the incubator for rarely longer than one half to two hours before use. The mixtures of alexin fractions, bacteria and phagocytes, were put into small test tubes (4 by ¼ inches) in which they could be easily shaken up, and were exposed to 38° to 40° C. in a water bath for from forty-five minutes to an hour. During this period they were shaken up three or four times.

After such incubation the tubes were gently centrifugalized in order that the leucocytes might be thrown down and relatively freed from extracellular bacteria. The supernatant fluid was poured off, the sediment reëmulsified in a few drops of salt solution, and this emulsion smeared upon slides,—a separate nipple pipette being used for each tube.

Considerable difficulty was encountered in obtaining well stained

preparations in which no gross errors in counting could occur. Satisfactory accuracy was attained in the earlier experiments by the use of Gram's stain after fixation by heat. By this technique the count could be assumed to be accurate, in that the contrast between bacteria and cells was sharp, but there was poor definition of the cells. In our later experiments we stained the slides in the following way with much more satisfactory results: The dried preparations were fixed in heat, and washed in considerable quantities of tap water, in order to remove the salt crystals. They were then stained with Gram's gentian violet for half a minute, and Gram's iodin for ten seconds. After this they were washed in alcohol until decolorized, and directly from the alcohol were plunged for fifteen to twenty seconds into Jenner's solution.

EXPERIMENTAL PART.

The tabulation of experiment I illustrates the plan of earlier experiments in which our results were negative, in that no opsonic action appeared either when the albumen fraction or the globulin fraction was used alone, or when the two were used together in quantities in which they produced the rapid and complete hemolysis of sensitized cells. Negative results were the rule in the first ten or fifteen experiments, and it was our inability to account for the fate of the opsonin during the splitting process that forced us to continue the tests.

EXPERIMENT I.

Preliminary Titration of Hemolytic Effects.[2]

Dialysis globulin fraction: 0.1 c.c. represents 0.1 c.c. of original serum.
Dialysis albumen fraction: 0.2 c.c. represents 0.1 c.c. of original serum.

1. Sensitized cells 0.5 c.c. + whole complement 0.1 c.c.
   = complete hemolysis in 5 min.
2. Sensitized cells 0.5 c.c. + globulin fraction 0.4 c.c.
   = no hemolysis.
3. Sensitized cells 0.5 c.c. + albumen fraction 0.6 c.c.
   = no hemolysis.
4. Sensitized cells 0.5 c.c. + globulin fraction 0.2 c.c. + albumen fraction 0.3 c.c.
   = complete hemolysis in 10 min.
5. Sensitized cells + salt solution = no hemolysis.

[2] The results in the hemolytic titrations were always checked up after twenty-four hours at room temperature, and if end-piece or mid-piece alone showed hemolysis, the experiment was discarded.

Phagocytosis Estimation.

	Experiment.	Phagocytic average. (100 cells.)
(*a*)	Bacteria	0.2 c.c.
	Whole complement	0.1 c.c.
	Leucocytes	0.2 c.c.
	Salt solution	0.5 c.c. = 20+
(*b*)	Bacteria	0.2 c.c.
	End-piece (albumen)	0.3 c.c.
	Leucocytes	0.2 c.c.
	Salt solution	0.3 c.c. = 2.1
(*c*)	Bacteria	0.2 c.c.
	End-piece (albumen)	0.5 c.c.
	Leucocytes	0.2 c.c.
	Salt solution	0.1 c.c. = 1.8
(*d*)	Bacteria	0.2 c.c.
	Mid-piece (globulin)	0.4 c.c.
	Leucocytes	0.2 c.c.
	Salt solution	0.2 c.c. = 0.8
(*e*)	Bacteria	0.2 c.c.
	Mid-piece	0.2 c.c.
	End-piece	0.3 c.c.
	Leucocytes	0.2 c.c.
	Salt solution	0.1 c.c. = 2.5
(*f*)	Bacteria	0.2 c.c.
	Leucocytes	0.2 c.c.
	Salt solution	0.6 c.c. = 0.2

In seeking for a possible fault in the technique to account for the complete loss of opsonic effects in the experiments, we were led to modify the procedure in two important points. To carry out completely the analogy between the phagocytosis of bacteria and the hemolysis of red cells, in their relation to complement, it seemed necessary to sensitize the bacteria by exposure to heated normal serum before carrying out the phagocytic experiment. It seemed possible also that with unsensitized as well as with sensitized bacteria there might be an error in our experiments depending on the use of excessive amounts of the globulin fraction (mid-piece), since an excess of this is known to inhibit hemolysis. Accordingly we planned our next experiment with these points in view, testing the opsonic action of the split products alone and together (*a*) upon normal bacteria, (*b*) upon normal bacteria treated with globulin

fraction (mid-piece), (*c*) upon bacteria treated with normal heated serum (sensitized), and upon sensitized bacteria treated with mid-piece, persensitized to follow the designation of Michaelis.

A few of these experiments follow.[3]

EXPERIMENT II.

The purpose of this experiment was to determine whether the previous exposure of bacteria to the globulin fraction rendered them more amenable to opsonic action by the albumen fraction than they were when the two fractions were added together. In each case preliminary hemolytic titrations were carried out as before, and phagocytic experiments were not done until end-piece and mid-piece were found entirely inactive alone, and active together. Experiments in which hemolysis appeared with end- or mid-piece alone by the next morning were not tabulated.

Dialysis albumen fraction; carbon dioxide globulin fraction.

The bacteria were emulsified in salt solution. Globulin fraction 0.2 c.c. was added to 1 c.c. of the emulsion, left together at 37.5° C. for 40 minutes, then thrown down in the centrifuge and reëmulsified in salt solution.

Experimental procedure.	Phagocytic average. Experiment (1913).		
	July 8.	July 10.	July 14.
(*a*) Bacteria+globulin fraction left 40 min., washed, then added albumen from 0.2 c.c.+leucocytes.....	20.0+	6.9	9.6
(*a'*) Bacteria+globulin fraction, left 40 min., washed, then added salt solution+leucocytes (control for (*a*) with same emulsion of bacteria).....	0.43	0.6	2.7
(*b*) Bacteria: Albumen fraction 0.2 c.c. Leucocytes.....	11.8	6.4	9.4
(*c*) Bacteria: Globulin fraction 0.1 c.c. Leucocytes.....	2.15	1.2	2.5
(*d*) Bacteria: Salt solution. Leucocytes.....	0.56	0.8	1.1
(*e*) Bacteria: Whole complement 0.1 c.c. Leucocytes.....	20.0+	12.0	13.5

The results of this experiment will be discussed below with those of experiments III and IV.

EXPERIMENT III.

The purpose of this experiment was to compare in one experiment the phagocytosis of bacteria treated with heated normal serum (sensitized) to that of

[3] The amounts of the albumen and globulin fractions used in the phagocytic experiment in which the two were recombined corresponded to those that gave the most prompt and complete hemolysis.

bacteria treated first with normal serum and then with the globulin complement fraction (persensitized) in the presence and absence of the albumen fraction.

Preliminary hemolytic titrations as before.

An emulsion of staphylococci in salt solution was treated with heated normal guinea pig serum for 30 minutes at 37.5° C. (sensitization). The bacteria were then thrown down in the centrifuge[4] and reëmulsified in salt solution. This emulsion was then divided into two parts, A and B. To A was added globulin fraction (mid-piece), 0.2 c.c. (to 1 c.c. of emulsion), and this mixture was left for 30 minutes at 37.5° C. Again the bacteria were thrown down and reëmulsified. We now had two staphylococcus emulsions, one treated with heated normal serum (sensitized), the other treated with this and also with mid-piece (persensitized). The two were equalized as far as possible and the following experiment was carried out. Since it was of course not possible to equalize exactly the two emulsions, it will be seen that we duplicated some of the controls.

Dialysis albumen fraction; dialysis globulin fraction.

Experiment.		Phagocytic average, July 25, 1913.
A (1)	Bacteria sensitized Albumen fraction Leucocytes	= 13.2
A (2)	Bacteria sensitized Salt solution Leucocytes	= 1.5
A (3)	Bacteria persensitized Albumen fraction Leucocytes	= 12.6
A (4)	Bacteria persensitized Salt solution Leucocytes	= 2.22
B (1)	Bacteria sensitized Albumen fraction Leucocytes	= 12.7
B (2)	Bacteria sensitized Globulin fraction Leucocytes	= 2.44
B (3)	Bacteria sensitized Globulin fraction Albumen fraction Leucocytes	= 9.0
B (4)	Bacteria sensitized Whole complement Leucocytes	= 20.0+

[4] In the centrifugations there was always a loss of bacteria, since they were never completely thrown down within thirty minutes by the centrifuge that we used.

EXPERIMENT IV.

July 26, 1913.

The purpose of this experiment was to compare normal, sensitized, and persensitized bacteria under the same conditions.

Hemolytic titrations were carried out as before.

Dialysis albumen fraction; carbon dioxide globulin fraction.

Substances added in phagocytic experiment (volumes equalized with salt solution).	Phagocytic average.		
	A. Normal bacteria.	*B.* Bacteria sensitized with normal heated serum.	*C.* Bacteria sensitized with heated serum, then exposed to globulin fraction (persensitized).
1. Albumen fraction alone	9.0	9.8 15.1[5]	15.3
2. Globulin fraction alone	0.5	—	—
3. Albumen fraction Globulin fraction	16.7	15.3	—
4. Salt solution	0.48	1.12	0.7
5. Whole complement	14.0	18.0	—

The results of these experiments indicate that the albumen fraction (end-piece) is the essential factor in determining normal opsonic action. From experiment II (*c*) (three separate measurements) it is seen that mid-piece alone may carry some slight opsonic properties, and, from a comparison of (*a*), (*b*), and (*c*) of the same experiment, that a coöperation of albumen and globulin fractions is more powerfully active than the albumen fraction alone. However, prolonged experience with experiments of this kind leads us to place little confidence in the significance of such slight differences of measurement, and we feel that the only positive conclusion that we can draw from experiment II is that bacteria may be powerfully phagocyted in the presence of end-piece or albumen fraction alone, under conditions in which this fraction has absolutely no action upon sensitized red cells. The same applies to experiments III and IV. Here again it is suggested that the sensitization of bacteria with heated serum and preliminary treatment with the globulin fraction may reënforce the opsonic action of the albumen portion. But the

[5] 15.1 is the count made with a different emulsion equalized optically to that used in the experiments with the normal bacteria. The discrepancy between this count and the other (9.8) shows that no conclusions can be drawn from the slight differences in degree of phagocytosis when the emulsions are equalized optically instead of representing equal parts of the same emulsion.

differences that indicate this again are slight and we can draw but one positive conclusion; namely, that the essential participant in the normal opsonic action is the end-piece, or albumen fraction.

In the experiments tabulated, as well as in a number of others, this result was unmistakable and definite. Yet for a long time we were not able to produce these results at will, many experiments showing, like the earlier ones, complete disappearance of the opsonic action after complement-splitting. When opsonic action could be determined at all it resided most powerfully in the end-piece. Never did it reside to more than a very slight degree in the globulin, or mid-piece, alone. When phagocytosis occurred after reconstruction of the complement by reunion of the fractions, it occurred also, and with nearly the same power under the influence of the albumen fraction alone. Often, however, it seemed to disappear entirely and was absent in all the tubes.

This inconsistency of results necessitated prolonged experimentation in which it was endeavored to ascertain the factor that determined our frequent negative results. Repeated hemolytic controls seemed definitely to exclude the possibility of deterioration of the complement during the process of separation as an explanation for this. We were finally led to seek the source of variation in alterations of the reaction of the serum during dialysis.

Liefmann and Stutzer (11) found, in 1910, that in bacteriolytic processes end-piece or albumen fraction alone was necessary for the exercise of the complementary function. On the basis of experiments by Braun (12) and further investigations of Liefmann and Cohn (13) they have retracted this view. Bronfenbrenner and Noguchi (14) in an extensive biochemical study of complement-splitting have come to the conclusion that in hemolysis, also, the end-piece alone is the active element, which, in the process of fractionation, is inactivated by change of reaction. It was their work particularly that caused us to focus our attention upon the reaction of our products, in experiments in which we confined ourselves entirely to the dialysis method. The following is a representative titration, which illustrates our general results; namely, that the albumen fraction obtained after dialysis is distinctly more acid than the original serum. Since the accurate titration of small quantities

is subject to considerable possibility of error, we requested Professor Howe, of the Department of Biological Chemistry, to check up our results. The following titration is one which was kindly done by him both with his own solutions and with those prepared by us.

TITRATION OF SERUM.

Fresh guinea pig serum was divided into two parts. One part was placed on ice; to the other (6 c.c.) 12 c.c. of distilled water were added. The mixture was placed in a new fish bladder dialyzing bag and dialyzed for 24 hours as usual against running distilled water. Two liters of the same water used for dilution and for dialysis were kept sealed with paraffin, so that no errors might arise from this source. The titrations tabulated were done with $N/250$ sodium hydrate, prepared by Dr. Howe, with phenolphthalein as an indicator. The dialyzed serum was pipetted out of the bag into a clean test-tube. The bag was washed out with this water, sufficient in amount to bring the total volume to 24 c.c. This was then centrifugalized until clear.

Titrations.

30 c.c. of distilled water	= 0.4 $N/250$ sodium hydrate.
Original serum 1 c.c. } 30 c.c. distilled water }	= 0.25 $N/250$ sodium hydrate.
The alkalinity of the serum to phenolphthalein	= 0.15 $N/250$ sodium hydrate.

Albumen dialysate equivalent to 1 c.c. of original serum brought to 30 c.c. with distilled water = 1.6 $N/250$ sodium hydrate.

Subtract water	=	0.4
The acidity of the dialysate	=	1.2 $N/250$
Gain in acidity during dialysis	=	1.35 $N/250$ acid.

It was evident therefore that the experiments carried out with the albumen fraction alone, both hemolytic and opsonic, were done under conditions of reaction different from those done with undialyzed complement. Our variable results could possibly be explained, therefore, on the basis of differences of reaction due to the various lots of distilled water used for dialysis, when these experiments were begun in California. There we had used water obtained from three different stills, without recording the source of the water in each case. With the water used in the laboratory at Columbia University, always from the same still,[6] uniformly negative results were obtained until we paid attention to the reaction. This was done in experiments of which the following tabulations are examples.

[6] This water was used in the above titrations.

EXPERIMENT V.

Dialysis Albumen Fraction.

Preliminary Hemolytic Titration.

December 6, 1913.

Whole complement 0.5 c.c. + sensitized cells 0.5 c.c. = complete hemolysis in 5 min.

Albumen fraction alone 0.6 c.c. + sensitized cells = no hemolysis in 24 hrs.

Globulin fraction 0.2 c.c. + sensitized cells = no hemolysis in 24 hrs.

Globulin fraction 0.2 c.c. + albumen 0.3 c.c. + sensitized cells = complete hemolysis in 15 min.

A. Hemolytic Experiment.

Albumen fraction (end-piece).	Acid or alkali made isotonic with 10 per cent. sodium chloride.[7]	Sensitized cells.	Salt solution.	Hemolysis.
1. 0.4 c.c. +	$N/350$ hydrochloric acid	0.4 c.c. +	0.5 c.c. + 0.2 c.c.	= very slight hemolysis.
2. 0.4 c.c. +	$N/350$ hydrochloric acid	0.2 c.c. +	0.5 c.c. + 0.4 c.c.	= no hemolysis.
3. 0.4 c.c. +	no acid or alkali	+	0.5 c.c. + 0.6 c.c.	= no hemolysis.
4. 0.4 c.c. +	$N/350$ sodium hydrate	0.2 c.c. +	0.5 c.c. + 0.4 c.c.	= no hemolysis.
5. 0.4 c.c. +	$N/350$ sodium hydrate	0.4 c.c. +	0.5 c.c. + 0.2 c.c.	= no hemolysis.
6. 0.4 c.c. +	$N/350$ sodium hydrate	0.6 c.c. +	0.5 c.c. + 0.0 c.c.	= no hemolysis.

B. Phagocytic Experiment.

Albumen fraction (end-piece).	Acid or alkali.	Leucocytic emulsion.	Bacterial emulsion.	Salt solution.	Phagocytic average.	Percentage of cells containing bacteria.
1. 0.4 c.c.+	$N/350$ hydrochloric acid	0.4 c.c.+	0.3 c.c.+	0.3 c.c.+	0.2 c.c.=0.18	12%
2. 0.4 c.c.+	N/350 hydrochloric acid	0.2 c.c.+	0.3 c.c.+	0.3 c.c.+	0.4 c.c.=0.49	23%
3. 0.4 c.c.	.....	+	0.3 c.c.+	0.3 c.c.+	0.6 c.c.=1.36	50%
4. 0.4 c.c.+	$N/350$ sodium hydrate	0.2 c.c.+	0.3 c.c.+	0.3 c.c.+	0.4 c.c.=4.9	78%
5. 0.4 c.c.+	$N/350$ sodium hydrate	0.4 c.c.+	0.3 c.c.+	0.3 c.c.+	0.2 c.c.=6.68	89%
6. 0.4 c.c.+	$N/350$ sodium hydrate	0.6 c.c.+	0.3 c.c.+	0.3 c.c.+	0.0 c.c.=4.5	55%

From experiment V it appears that the alteration of reaction does not activate the hemolytic properties of the albumen fraction upon sensitized cells. This is contrary to the results of Bronfenbrenner and Noguchi. The slight hemolysis in tube A (1) we attributed to the acid, and this we found to be the case, as will be seen in the next experiment. The result in the opsonic experiment, however, showed that a restitution of alkalinity restored opsonic power to the albumen fraction, apparently an optimum being reached with the addition of 0.4 of a cubic centimeter of an $N/350$ sodium hydrate solution to 0.4 of a cubic centimeter of the end-piece.

[7] The acid and alkali solutions were made isotonic with 10 per cent. salt solution.

The table (experiment VI) that follows is similar except that at the same time we carried out an attempted restitution of complement (C) by reunion of the fractions, as in the earliest experiments.

EXPERIMENT VI.

Preliminary Hemolytic Titration.

A.

1. Whole complement 0.5 c.c. + sensitized cells 0.5 c.c. = hemolysis in 8 min.
2. Albumen fraction alone 1 c.c. + sensitized cells = no hemolysis in 24 hrs.
3. Albumen fraction alone 3 c.c. + sensitized cells = no hemolysis in 24 hrs.
4. Globulin fraction alone 0.4 c.c. + sensitized cells = no hemolysis in 24 hrs.
5. Globulin fraction 0.2 c.c. + albumen fraction 0.3 c.c. + sensitized cells = hemolysis in 20 min.
6. Globulin fraction 0.3 c.c. + albumen fraction 0.5 c.c. + sensitized cells = hemolysis in 10 min.

B.

Serum constituents.	Leucocytic emulsion.	Bacterial emulsion.	Salt solution.	Result. Phagocytic average.	Result. Percentage of cells containing bacteria.
1. Whole complement	0.1 c.c. +	0.3 c.c. +	0.3 c.c. + 1.0 c.c. =	3.25	80%
2. Salt solution	1.1 c.c. +	0.3 c.c. +	0.3 c.c. + 0.0 c.c. =	0.5	26%
3. Globulin fraction (mid-piece)	0.4 c.c. +	0.3 c.c. +	0.3 c.c. + 0.7 c.c. =	0.36	18%
4. Globulin fraction (mid-piece) Albumen fraction (end-piece)	0.3 c.c. + 0.5 c.c.	0.3 c.c. +	0.3 c.c. + 0.3 c.c. =	0.5	25%

Hemolytic Experiment.

C.

Albumen fraction (end-piece).	Acid or alkali.	Sensitized cells.	Salt solution.	Result.
1. 0.5 c.c. +	$N/350$ hydrochloric acid 0.6 c.c. +	0.5 c.c. +	0.0 c.c. =	hemolysis moderate in 12 hrs.
2. 0.5 c.c. +	$N/350$ hydrochloric acid 0.4 c.c. +	0.5 c.c. +	0.2 c.c. =	very slight hemolysis in 12 hrs.
3. 0.5 c.c. +	$N/350$ hydrochloric acid 0.2 c.c. +	0.5 c.c. +	0.4 c.c. =	no hemolysis.
4. 0.5 c.c. +	$N/350$ hydrochloric acid 0.0 c.c. +	0.5 c.c. +	0.6 c.c. =	no hemolysis.
5. 0.5 c.c. +	$N/350$ sodium hydrate 0.2 c.c. +	0.5 c.c. +	0.4 c.c. =	no hemolysis.
6. 0.5 c.c. +	$N/350$ sodium hydrate 0.4 c.c. +	0.5 c.c. +	0.2 c.c. =	no hemolysis.
7. 0.5 c.c. +	$N/350$ sodium hydrate 0.6 c.c. +	0.5 c.c. +	0.0 c.c. =	no hemolysis.
8. Control—acid alone	0.6 c.c. +	0.5 c.c. +	0.0 c.c. =	strong hemolysis in 12 hrs.
9. Control—alkali alone	0.6 c.c. +	0.5 c.c. +	0.0 c.c. =	no hemolysis.

Phagocytic Experiment.

D.

Albumen fraction (end-piece).	Acid or alkali.	Leucocytic emulsion.	Bacterial emulsion.	Salt solution.	Phagocytic average.	Percentage of cells containing bacteria.
1.	0.5 c.c.+N/350 hydrochloric acid	0.6 c.c.+	0.3 c.c.+	0.3 c.c.+0.0 c.c.	0.36	16%
2.	0.5 c.c.+N/350 hydrochloric acid	0.4 c.c.+	0.3 c.c.+	0.3 c.c.+0.2 c.c.	0.18	14%
3.	0.5 c.c.+N/350 hydrochloric acid	0.2 c.c.+	0.3 c.c.+	0.3 c.c.+0.4 c.c.	0.32	22%
4.	0.5 c.c.+N/350 hydrochloric acid	0.0 c.c.+	0.3 c.c.+	0.3 c.c.+0.6 c.c.	0.38	26%
5.	0.5 c.c.+N/350 sodium hydrate	0.2 c.c.+	0.3 c.c.+	0.3 c.c.+0.4 c.c.	1.98	64%
6.	0.5 c.c.+N/350 sodium hydrate	0.4 c.c.+	0.3 c.c.+	0.3 c.c.+0.2 c.c.	3.4	80%
7.	0.5 c.c.+N/350 sodium hydrate	0.6 c.c.+	0.3 c.c.+	0.3 c.c.+0.0 c.c.	4.01	78%

We may summarize this experiment as follows:

A. By dialyzing guinea pig serum against distilled water for a sufficient length of time a supernatant albumen fraction was obtained which by itself had no hemolytic properties even when added in quantities of three cubic centimeters (corresponding to one cubic centimeter of whole serum) to highly sensitized cells. The precipitated globulins (mid-piece) after two reprecipitations were similarly inactive for hemolysis. But together in suitable amounts these two fractions cause prompt hemolysis.

B. Similarly tested for opsonic properties neither of these fractions possessed any phagocytosis-enhancing powers, nor did they show this when put together in amounts in which they were hemolytically active.

C. When the albumen fraction alone was tested for hemolytic properties in a series in which small amounts of acid or alkali had been added, no reactivation, as found by Bronfenbrenner and Noguchi, could be demonstrated, at least in the quantities employed by us (equivalent to 0.16 of original serum).

D. The albumen fraction (end-piece) alone showed definite and progressively increasing opsonic activity as small amounts of alkali were added, approximately restoring the conditions of reaction prevailing in normal guinea pig serum. Figures 1 and 2 illustrate these relations.

SUMMARY AND CONCLUSIONS.

Our experiments show that the albumen fraction, or end-piece, obtained by the dialysis of normal guinea pig serum possesses defi-

nite opsonic action. This action is often almost equal to that residing in the unfractionated alexin. It is evident, however, only if the reaction maintained during the experiments approximates that of the original serum. By the addition of small quantities of a weak sodium hydrate solution to the dialyzed serum we have been able to bring back opsonic action which was not evident in the same end-piece if simply rendered isotonic.

Although our attention was called to the question of reaction by the work of Bronfenbrenner and Noguchi, like Liefmann, we have been unable to reactivate the hemolytic function of end-piece by alteration of reaction. Our experiments suggest that the opsonic action of the albumen fraction is enhanced by preliminary sensitization of the bacteria with heated normal serum and by persensitization of such bacteria with the globulin fraction. However, we cannot be positive of this, since the slight differences of phagocytic counts upon which such an opinion can be based, fall within the limits of what we consider our experimental error.

The fact that the albumen fraction can exert opsonic activity upon bacteria but cannot hemolyze blood cells seems to us particularly interesting in the light of the fact that alexin can be absorbed by unsensitized bacteria but not by similarly untreated blood cells. The literature upon the relation of the alexin fractions to bacteria and the bactericidal effect is confusing in that contradictory results have been obtained by other workers. We are studying this phase of the problem with particular attention to the alkalinity or acidity under which the reactions are carried out.

We think that our experiments do not point to a differentiation of normal opsonin from alexin, but we believe they indicate that the so called end-piece can enter to a slight extent into non-specific relationship with unsensitized bacteria, and it is therefore active, whereas it cannot enter into a similar relation to unsensitized cells. This conception, however, is tentatively made, since we are studying further the non-specific absorption of alexin or complement by unsensitized bacteria.

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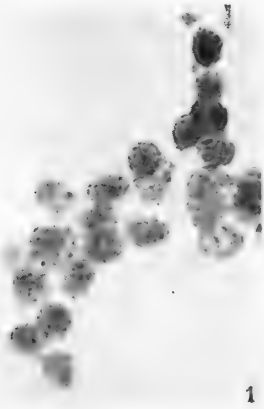

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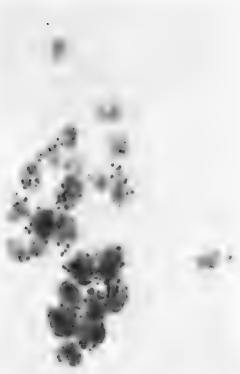

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EXPLANATION OF PLATE 40.

FIG. 1. Field from preparations in which 0.4 c.c. of the albumen fraction was used with bacteria and leucocytes.

FIG. 2. Same end-piece, or albumen fraction, as figure 1, with same bacteria and leucocytes, with the addition of 0.4 c.c. $N/350$ sodium hydrate.

OBSERVATIONS ON A STRAIN OF SPIROCHÆTA PALLIDA ISOLATED FROM THE NERVOUS SYSTEM.*

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PLATES 41 AND 42.

Our knowledge of *Spirochæta pallida* is now entering on the stage of differentiation of strains, which has already been reached in the study of many other pathogenic microörganisms. In parasitology in general, the single, original organism is soon replaced by a group of related organisms and this group is developed as a result of investigation in two directions. On the one hand relationships are discovered with outlying organisms, and on the other hand the original organism itself is resolved into a number of varieties so that the clear cut outlines of our first views give way, on closer observation, to a confusing series of gradations. In the case of *Spirochæta pallida* the same change may be noted. Soon after the discovery of the pallida, an outlying organism was found in *Spirochæta pertenuis,* which, while a distinct organism, undoubtedly belongs in the pallida group (1). On the other hand the pallida itself, according to Noguchi, consists of three different forms, the thick, the thin, and the medium form, and, what is more significant, the thick form produces characteristic lesions in the rabbit, different from those produced by the thinner forms (2).

While the work of differentiating strains is usually confined to biological characters, the explanation of clinical variations is the goal of scientific medicine. From the clinical side there has long come a strong suggestion of the existence of strains of pallida, especially of a neurotropic strain. Clinical evidence pro and con has been cited by many authors (3). Experience has shown that

* Received for publication, January 22, 1914.

clinical suggestions based on the observations of the patient alone are often fallacious on account of the many factors involved, but at the same time they form the starting point for experimental work. In the case of some organisms, the typhoid bacillus for example, the lack of a suitable experimental animal has prevented a satisfactory demonstration of the existence of mild or virulent strains, etc. In experimental syphilis, however, we have in the rabbit an animal by which, I think, certain strains can be clearly differentiated.

In this paper I propose to give some evidence on which I believe it is possible to differentiate a strain isolated from the nervous system. Whether the strain should be called neurotropic or simply highly invasive will be discussed later. As far as it is known no observations have been made on a similar strain. Most of the work in experimental syphilis has been carried out with strains isolated from chancres or mucous patches, and the clinical potentialities of these strains are entirely unknown (4). Hence it is not safe to draw clinical conclusions from the lesions in the animal. Again in the only other instance, before ours, in which a strain has been isolated from the nervous system, the experimenter simply made the statement that a typical lesion was produced in one transfer (Hoffmann (5)). Since we isolated this strain, another one has been secured by Uhlenhuth and Mulzer under similar circumstances, but no detailed report on it has been made (6). The results of experimental work in paresis will be referred to later, but it may be said here that no definite conclusions can yet be drawn except that it is difficult to transfer and to continue the strain in the rabbit. If there is any special strain of pallida, the proper way to study it would seem to be to work back from the clinical condition to the experimental animal and to carry on the strain long enough to determine its peculiar characteristics. The subject of strains of pathogenic microörganisms is, of course, a complicated one, as it calls for consideration of variations in both the host and the parasite. In this paper only variations in the parasite are considered and the conditions of the host have been made as uniform as possible by carrying the strain through a large series of animals. Fourteen transfers have been made covering a period of eighteen months, and lesions have been studied in twenty animals. Certain con-

stant characteristics have been noted and these have been compared with lesions developing in four other strains isolated from mucous patches or chancres, one of which has been carried on for four years (7).

The strain in question was isolated by Dr. Hough and myself from the spinal fluid of a case of nervous relapse following salvarsan in the secondary stage of the disease (8). The patient had definite primary and secondary symptoms, and pains in the muscles and joints probably indicated that the nervous system was already involved (9). Two intravenous injections of salvarsan were given and the symptoms rapidly cleared up. Five months later the patient developed a definite psychosis with partial paralysis of the right side of the body. The cells in the spinal fluid reached 840 per cubic millimeter and the organism was recovered by injecting three cubic centimeters of whole spinal fluid into a rabbit's testicle. The case was a typical one of nervous relapse in the secondary stage following inadequate treatment. According to our theory of the condition, the nervous system must have been already invaded during the early secondary period, and the patient would probably have suffered from nervous syphilis later.

In morphology the strain corresponds to Noguchi's thick type. While characteristic of the pallida in its sharp regular twists, the body is distinctly thicker than that of many other specimens which have been examined. As a result the curves are not so deep as in the thinner varieties. In Levaditi sections the thickness is especially noticeable, being almost like that of the coarser spirochætes, such as *Spirochæta refringens*. It is recognized that in dealing with such a minute organism as the pallida, too much weight should not be given to slight differences in form, but a constant difference is of some significance especially when accompanied by distinct lesions, as in the present instance.

Noguchi has noted that on inoculation the thick form produces hard nodules almost cartilaginous in consistence and sharply defined from the surrounding tissues. This description is suggestive in considering the present strain. On inoculation of the scrotum or testicle, a hard, discrete nodule develops and the rim retains this character while the center becomes distinctly necrotic (figure

1). Spirochætes are found in abundance in the rim but are scarce in the center of the lesion. The necrosis of the core is not due to mixed infection as no extraneous organism can be demonstrated by cultures. The sort of lesion developed by this strain is easily differentiated from the diffuse, edematous lesion which is most frequently produced by inoculation from chancres and mucous patches. With a medium type of organism, which has been carried on for four years, the lesion always grades off into the surrounding testicular tissue. On intravenous inoculation the same sort of hard, discrete lesion is produced in the testicle and scrotum. In connection with the hardness of the nodule, it is suggestive that nervous relapses have been most frequently noted in patients who have shown the papular form of secondary eruption rather than the macular (10). This clinical finding is probably to be explained by the strain of spirochæta concerned. Histologically the rim consists of a dense collection of lymphoid cells, while the center is composed of a loose connective tissue with few cells and shows evidences of degeneration.

Aside from a characteristic lesion, a characteristic location has been noted, namely, on the scrotal side of the tunica vaginalis. The lesion might be called an internal chancre. The skin surface of the scrotum and the testicle may not be at all affected, but a definite nodule will develop between them. This lesion has resulted both from local inoculation of the testicle and from intravenous injection. A similar localization has been noted in experimental syphilis with ordinary strains and may have no significance, but its occurrence is quite constant with this strain.

The third feature about this strain is a short incubation period which averaged eighteen days in testicular or scrotal inoculations. No great emphasis is laid on this fact, except when it is taken in consideration with the other characters. In Noguchi's two thick forms isolated from protracted chancres, the incubation period was five or six weeks.

The fourth and most striking feature is the generalization of this strain with the production of lesions remote from the site of inoculation. Generalization of the infection from a local inoculation has been noted occasionally by Uhlenhuth and Mulzer and

by others. Occasionally the opposite testicle is affected, rarely the eye. With the present strain one half of the animals observed have shown this generalization. The lesions have been chancres in the opposite scrota, nodules in the opposite testicle, but, most noticeably, keratitis and lesions of the eyelid. The average incubation of the first lesion was fifteen days, and of the secondary lesion forty-five days. In other words, about one month has elapsed before the generalization has become evident.

GENERALIZATION TO THE EYELIDS.

Rabbit 5/8.—Nov. 19, 1912. Right testicle; inoculation with material taken in pipette from testicle of rabbit 5/6; 5th transfer.

Dec. 2, 1912. Definite nodule present (13 days).

Jan. 6, 1913. Large nodule present in right testicle. Left scrotum shows small chancre (48 days).

Jan. 13, 1913. Multiple nodules present on the edge of upper and lower eyelids. Spirochætæ numerous (55 days).

Rabbit 5/26.—Oct. 22, 1913. Both scrota inoculated with material taken in pipette from nodule of testicle of rabbit 5/24; 13th transfer.

Oct. 31, 1913. Chancre present, right side (9 days).

Nov. 14, 1913. Chancre present, left side (23 days).

Dec. 6, 1913. Both chancres nearly healed; multiple nodules present on margin of eyelids of both eyes. Spirochætæ positive (45 days) (figure 2). Nodule present in left epididymus major (45 days). Spirochætæ positive.

The developments in rabbit 5/26 are especially striking since no inoculation was made in the testicle. After a simple subcutaneous inoculation, which was followed in nine days by a chancre, there developed in forty-five days multiple nodules in the eyelids and a lesion of the testicle, all rich in spirochætæ. Generalization of the virus can often be secured by intravenous injections especially after a strain has passed through a series of animals, but generalization following a simple cutaneous inoculation is an unusual and striking occurrence, and it has been observed so often with this strain that it must be regarded as characteristic.

GENERALIZATION TO THE CORNEA.

Rabbit 5/9.—Dec. 10, 1912. Right testicle inoculated with material taken in pipette from testicular lesion of rabbit 5/8; 6th transfer.

Dec. 30, 1912. Nodule positive in epididymus major (20 days).

Jan. 20, 1913. Nodule positive; testicle removed; large nodule with hard rim and necrotic center present. No mixed infection.

Feb. 28, 1913. Both eyes show diffuse keratitis with injection of corneal margin and pannus (80 days after inoculation, 39 days after removal of testicle).

Apr. 2, 1913. Left eye excised. Spirochætæ positive in scraping from cornea, and present in large numbers in Levaditi section (figure 3).

On intravenous injection in adult animals the same results are readily produced.

CHOROIDITIS.

In the course of the examination of the lesions of the cornea, a new lesion of experimental syphilis in the eye has been discovered by Dr. Louis Green, by the use of the ophthalmoscope; namely, a definite choroiditis. The condition has been noted in a number of animals with this strain after local inoculation, either with or without other lesions of the eye. The condition closely resembles that found in the human eye and consists of hemorrhagic points generally diffused in the lower pole of the eye. A detailed report of this lesion will be made elsewhere, but after careful study of a large number of normal eyes there is no doubt about its occurrence. It has never been observed with other strains except after intravenous injections. In one case the choroid was removed and was injected into a rabbit's testicle, but no lesion resulted. Treatment with salvarsan does not produce much improvement in the lesion.

As a further evidence of the invading power of this strain, it may be mentioned that local inoculation of the anterior chamber of the eye has always resulted positively, while with other strains my results have been slight and uncertain. The occasional occurrence of any one of the generalized lesions mentioned might be disregarded, but their occurrence in one half of the animals observed seems to me clear evidence that we are dealing here with a distinct strain, whose chief characteristic may be called its invasive power. It might also be called more virulent than other strains, or more pathogenic, but these terms are more indefinite than the one used.

PARESIS.

Now that it has been shown that paresis is "parenchymatous" syphilis of the nervous system, it becomes of interest to know the characteristics of the strain, if there is one, which produces this

disease. Unfortunately the strain seems difficult to transfer to rabbits. Noguchi succeeded in 2 out of 36 animals inoculated from 6 cases in producing lesions with spirochætæ. In one case they were present in few numbers in a section, and in one case they were found in a chancre more abundantly, but the second transfer resulted in only a trivial lesion (11). No mention is made of the type of organism. Berger has reported that after prolonged search he found a few spirochætæ in 3 slight lesions out of 20 inoculations (12). No attempt was made to continue the strain. Dr. Hough and I have made 5 inoculations of spinal fluid from 5 cases of paresis, all with negative results. We have made 16 inoculations of brain substance from 8 cases and have had slight success in 2 instances (13). In one case a slight testicular lesion was followed by a marked double keratitis, and inoculation of the cornea resulted in another slight lesion of the testicle with a definite choroiditis. Inoculation of the choroid resulted in a unilateral keratitis in the third generation, but we have been unable to demonstrate spirochætæ in the lesions or to continue the strain. In the other case a small nodule developed in the testicle after thirty-one days, but spirochætæ could not be found and transfers were unsuccessful. The only conclusion to be drawn from this work so far is that the local lesions which result from the inoculation of the brain substance in paresis are slight and contain few organisms. This finding is in marked contrast to the well marked lesion, rich in spirochætæ, which develops with the strain isolated from the case of nervous syphilis. The only resemblance between the two strains is the apparent generalization of our first strain from a paretic in the form of keratitis. The natural assumption is, of course, that in paresis the spirochætæ become modified by their long parasitization in the human brain and lose some of their power to thrive elsewhere. In this way an originally highly invasive strain might not appear so in a few transfers.

DISCUSSION.

The evidence presented above seems to me to indicate that the strain in question is a distinct one and it is characterized chiefly by its invasive power. No evidence has been found in the rabbit of any especial neurotropic power, and inoculation of a monkey's

brain was negative. By highly artificial means, such as the intravenous injection of a rich emulsion of spirochætæ, I have obtained in one case, with a generalized eruption of the skin, some symptoms of involvement of the nervous system and some slight infiltration in the meninges, but my object in general has been to imitate natural conditions as far as possible with the results given above. I believe that these experimental results may have a clinical equivalent. There is some evidence that the nervous system is more resistant to *Spirochæta pallida* than other parts of the body, and only the more active strains may be able to establish themselves there. In other words, the invasive power of the parasite may be of more importance in syphilis of the nervous system than its affinities. An affinity seems to exist in the sexual organs, and all strains might be called genitotropic, as, after intravenous injection, they invariably locate in the sexual organs whether they do elsewhere or not. But when, after local inoculation, the organism leaves the site of predilection and establishes itself in other tissues, a new factor seems to be introduced and this new factor may be called its invasive power.

Variations in this power seem to afford ground for classifying the members of the pallida group in a series of gradations. *Spirochæta pertenuis* must certainly be classed in the pallida group, but its invasive power is much less than that of the pallida. Yaws is known clinically as a non-venereal, non-hereditary, skin disease. While it is a general disease in which the spirochætæ are distributed by the blood, it also spreads by auto-inoculation in the skin. And following the septicemic stage, the organisms appear always to settle in the skin and not to penetrate the other tissues, such as the nervous system. In the rabbit the pertenuis produces a constant and characteristic lesion of the scrotum consisting of a large, edematous chancre-like lesion which is distinct from the ordinary chancres seen in experimental syphilis (figure 4). On intravenous injections, lesions of the skin of the head are produced which are also distinctive (14). *Spirochæta pertenuis,* therefore, might be put at one end of the series as affecting chiefly the skin. The strain in question might be put at the other end of the series from a point of invasive power in experimental animals and in man. Other

strains of the pallida might be given an intermediate position according to their clinical and experimental characters. If this idea is correct, it is not unreasonable to imagine that in the course of time, an examination of the type of organism in a chancre may be used for prognosis. In this discussion the condition of the host has been intentionally ignored because it is desired to emphasize a definite character in the parasite. The so called resistance of the host must, however, be considered in the last analysis, as it might lessen or accentuate the original characters of the strain, but it could not entirely change them.

SUMMARY.

A strain of *Spirochæta pallida* isolated from the nervous system has shown the following constant characters: (1) thick form; (2) the production of hard, well demarcated lesions with necrotic centers; (3) a characteristic location of the lesion; (4) a short incubation period; (5) a tendency to generalize with lesions of the skin and eye, following local inoculation of the testicle and scrotum.

It is suggested that these characters constitute a definite strain of highly invasive power and that a classification of members of the pallida group may be made in accordance with this power.

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FIG. 1.

FIG. 2.

(Nichols: Observations on *Spirochæta pallida*.)

FIG. 3.

FIG. 4.

(Nichols: Observations on *Spirochæta pallida*.)

EXPLANATION OF PLATES.

PLATE 41.

FIG. 1. Type of lesion produced by subcutaneous inoculation of the scrotum with a strain from the nervous system; hard edge with completely necrotic center.

FIG. 2. Nodules on the margin of the eyelids of rabbit 5/26 (48 days after scrotal inoculation). Spirochætæ numerous.

PLATE 42.

FIG. 3. *Spirochæta pallida* in the cornea of rabbit 5/9; Levaditi section, × 1,300 (80 days after testicular inoculation).

FIG. 4. Typical lesion of the scrotum produced by subcutaneous inoculation of *Spirochæta pertenuis;* large, edematous lesion; superficial necrosis.

A NOTE ON NITROUS OXIDE AS AN ANESTHETIC IN ANIMAL EXPERIMENTATION.*

By DAVID H. DOLLEY, M.D.

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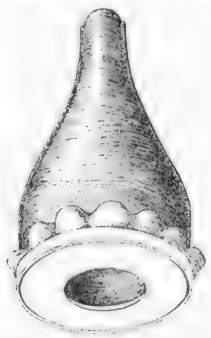
As far as the writer can discover from the literature and from personal inquiries, nitrous oxide, now commonly used in surgery, has not been applied as a routine in animal experimentation.

Its use in this laboratory has been limited to the dog, though there is no reason why it can not be extended to other animals. The writer was led to investigate its possibilities in the hope that it would be useful in simple and short operations, such as intravenous administration of drugs for later results, dressings which demand an anesthetic, etc. A test quickly showed that above all it possesses an inimitable value as a preliminary anesthetic, a value



TEXT-FIG. 1. Head mask for administration of gas to animals.

so great that it is worth the initial expense for that alone. For an ether anesthesia, a preliminary dose of morphin is apparently inferior to nitrous oxide as regards the ease of handling the animal.

The Gatch nitrous-oxide-ether-oxygen apparatus has been used

* Received for publication, January 23, 1914.

for administration.[1] It was necessary to devise a suitable head mask, which is represented in the accompanying text-figure. It consists of a cone ten inches in length and five inches in inside diameter at its base, made of fairly thick harness leather. The seams are paraffined inside and out to make it air-tight. The smaller orifice of the cone fits over the orifice of the tube conveying the gases from the apparatus, and the connection again is made air-tight by rubber bushing glued around the inside of the cone's orifice. The tube slips in and out with ease, yet the compressible rubber holds it firmly. The larger orifice is kept distended and circular by a ringlet of brass riveted just inside the opening. A cuff of rubber sheeting is fitted over the larger orifice and has a circular opening for the dog's nose. The cuff is held in place by an outside ring of brass, which slips on tightly. The cuff can therefore be removed from the cone at will, and several with different sizes of opening may be provided to fit the individual case. Cast off automobile inner tubing has been found to combine sufficient strength with the needed elasticity. This contrivance, devised to try out the apparatus, at least offers a tested suggestion for one of better construction and possibly of metal. The important feature is the cuff of rubber, which, with proper size of opening, has been found to fit over a dog's head practically in an air-tight way.

The convenience and ease of handling which the Gatch apparatus promotes may be briefly indicated by a summary of the records on twenty-one dogs. When the cone is applied and the gas turned on, it is slightly more usual for the animal to hold its breath. The average time required from the moment of admitting the gas until the animal becomes perfectly limp was one minute and eight seconds. The maximum time was two minutes and ten seconds, during which the animal held its breath for one minute and ten seconds. The average time of actual inhalation, from the first breath to complete anesthesia, was forty-nine seconds. The maximum duration was one minute and thirty seconds, and the minimum thirty seconds. In only the one instance just mentioned as the maximum has it been over sixty seconds. The time record, however, gives only a partial idea. Without the local irritation of ether and its physiolog-

[1] Gatch, W. D., *Jour. Am. Med. Assn.*, 1910, liv, 775; 1911, lvii, 1593.

ically excitant effect, struggling is reduced to a minimum. In fact many animals do not struggle at all, but, possibly after holding the breath for a few moments, pass quietly to deep inhalations. All the objectionable features of primary ether anesthesia are eliminated. The record embraces dogs of all types, ages, and sizes, including five large animals weighing over twenty kilograms. By this method the largest animal has been handled like the smallest one, and readily by two men.

In this apparatus the factor of rebreathing is involved. This question as well as the possibilities of administration are fully discussed in papers by Gatch.[2] It is sufficient to say that with proper management no deleterious effects have been noted from rebreathing. To have three possibilities of instantaneous shift of administration combined within one apparatus, namely, nitrous oxide, ether, and oxygen, is not infrequently advantageous. It seems of value to mention only a few details of application to the dog, as these are for the most part obviously apparent. It has become our practice to turn on the ether as soon as the animal breathes the nitrous oxide deeply. As the animal relaxes the valve box is quickly shifted to in and out breathing of the gas to empty the bag, and is then changed to to and fro rebreathing of ether and oxygen or of ether and air. Air and ether are mentioned because it has been found possible to get essentially the same results as circumstance requires by inflating the bag with air in the place of oxygen by a connection with a foot pump, which is a considerable economy. In either case the shift requires careful management to hold the anesthesia, as the animals come out quickly. Occasionally more nitrous oxide is necessary. Usually the initial bagful of nitrous oxide suffices. If the animal be subjected too long to nitrous oxide alone, fresh air, by disconnecting the tube, and perhaps a compression or two of the chest are sufficient to restore respiration. Our only fatality from this cause was the first experiment. If any objection is raised in regard to an admittedly forced administration of both nitrous oxide and ether at the beginning, it is not a practical one, and at the worst the anoxyemia and overconcentration of ether vapor can be no greater than they are by the usual cone method.

[2] Gatch, W. D., *loc. cit.*

The usefulness is not limited to preliminary anesthesia. If desired, as in experiments for recovery, a perfectly even anesthesia with ether may be maintained through the mask, as is proved by records of blood pressure and respiration; or the apparatus may be used only for the preliminary preparation for other ways of administration. It has also been shown to be feasible to maintain anesthesia with nitrous oxide and oxygen. Moreover, the student becomes familiar with the use of an apparatus which is a close imitation of current surgical procedure. Finally, after the initial cost, it is economical. The animal inhales all the ether given, the oxygen can be reserved for an emergency, and, as stated, one bagful of nitrous oxide has been the average per dog.

SUMMARY.

Nitrous oxide, administered by means of the Gatch[3] apparatus, as a preliminary anesthetic to ether has been found to be superior for the dog in rapidity of action, in promotion of ease of handling, and in absence of disorder; the anesthesia may be maintained solely by nitrous oxide in combination with oxygen; it is economical; and its use is instructive to students from closer imitation of current surgical methods.

[3] The Gatch apparatus is at present manufactured by the Charles Willms Surgical Instrument Company, Baltimore.

THE RAPIDITY OF THE INVOLUTION OF ACTIVE THYROID HYPERPLASIAS OF BROOK TROUT FOLLOWING THE USE OF FRESH SEA FISH AS A FOOD.*

By DAVID MARINE, M.D.

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In a recent paper (1) the effect of certain foods on the production and prevention of goitre in brook trout (*Salvelinus fontinalis*) was described and it was shown that a continuous diet consisting only of hog's liver and heart[1] quickly (in one to two months) induces well marked thyroid hypertrophy and hyperplasia, and that when hashed fresh sea fish alone was substituted, further hyperplasia was prevented, and any existing thyroid hyperplasia involuted to its colloid or quiescent state.

In the present communication will be given data concerning the rapidity of the involution following the substitution of the fish diet, and the time limits within which it is completed, and also, for comparison, the time limits of the involution induced by other means in Lake Erie pike, in brook trout, and in mammals (dogs).

The fish used in these observations were taken from the regular stock hatched about January 15, 1913, and distributed to the ponds about July 1. They had, therefore, in common with the entire 1913 hatch, been fed with finely divided hog's liver for the first few months (four to five), and after that with a mixture of heart muscle and liver. I arbitrarily chose fish from pond 8,[2] since complete examinations made in August of all the 1913 hatch, both those living in the troughs and those in the ponds, showed approximately the same degree of thyroid hyperplasia.

* Received for publication, February 7, 1914.

[1] The food is but one of a complex of interacting factors, among which are overcrowding, and relatively insufficient water supply, with its diminished oxygen and excess of excreta contents. The food, however, is believed to be the major single factor.

[2] For the plan of the hatchery see Marine, D., *Jour. Exper. Med.*, 1914, xix, 71.

The experiments were conducted as follows: On September 28, 1913, the food of all the fry of 1913 was changed to fresh butter fish (*Stromateus triacanthus*) given once daily except for the weekly fast day. Eight fish were taken from pond 8 on this date as controls, and four fish were removed every seventh day for sixty-three days following. The data are presented in the following tabulation.

The eight fish taken as controls at the beginning of the experiment have uniformly hyperplastic thyroids listed as "marked hyperplasias," comparable and similar in all essentials to those present in the fry of corresponding ages in 1909, 1910, 1911, and 1912. The strain of fish and the food (hog's liver and heart) have also remained constant during these years. The distribution of the thyroid tissue is approximately the same in all the forty-four fish used. In all cases the thyroid follicles have filled the thyroid area about the aorta and between the 1st and 3d gill arches, have extended up to the pharyngeal mucosa, have invaded the bone and muscles, and in most cases have extended through to the skin adjacent to the ventral attachments of the 1st and 2d gill arches.

Specimens taken seven days after the feeding of fish was instituted show slight but distinct changes in the thyroid toward involution. The follicular spaces are more distinct and contain some stainable colloid. The epithelium is slightly changed, and the stroma and vascularity appear unmodified, and, according to the arbitrary standards I have used, such a histological appearance would be designated "colloid-moderate-marked hyperplasia."

The fourteen day specimens are listed as "colloid-moderate hyperplasias," *i. e.,* the stainable colloid is more abundant, the follicular spaces better defined, and the epithelium has shrunken from high columnar to low columnar.

The twenty-one day specimens show still further accumulations of colloid in the follicles, and the lining epithelium has shrunken to the cuboidal form. These specimens are listed as "colloid-early hyperplasias."

Specimens removed twenty-eight days after the fish feeding was instituted show still further shrinking of the epithelium, and the colloid content is now nearly uniform and homogeneous. Vacuoles

TABLE I.

Pond No.	Age in months.	Date taken.	Food and length of time fed.	Condition of thyroid.		Remarks.
				Extent of overgrowth.	Classification.	
8	8+	Sept. 28, 1913	Hog's liver for 1st 4 mos. of life; then hog's liver and heart	Entire subpharyngeal area infiltrated, including bone and muscle	Marked hyperplasia	This is an example of 8 controls. All have approximately the same degree of hyperplasia.[3] Colloid absent.
8	8+	Oct. 5, 1913	Whole butter fish only, beginning Sept. 28, 1913, 1 wk.	Entire subpharyngeal area infiltrated, including bone and muscle	Colloid-moderate-marked hyperplasia (beginning involution)	This is an example of 4 specimens examined. All have approximately the same thyroid condition. Colloid appears in follicles.
8	8+	Oct. 12, 1913	Butter fish only for 2 wks.	Thyroid area filled, extending to pharyngeal mucosa, in bone and muscle	Colloid-moderate hyperplasia (involuting)	This is an example of 4 specimens examined. All have approximately the same thyroid condition. Colloid in fair amounts in all follicles. Epithelium low columnar.
8	9	Oct. 19, 1913	Butter fish only for 3 wks.	Entire subpharyngeal area filled, extending to pharyngeal mucosa and skin and gills, in bone and muscle	Colloid-early (involuting)	This is an example of 4 specimens examined. All have approximately the same thyroid condition. Colloid in all follicles fairly homogeneous. Epithelium cuboidal.
8	9+	Oct. 26, 1913	Butter fish only for 4 wks.	Entire subpharyngeal area filled, extending to pharyngeal mucosa, bone, and muscle	Colloid-early (approximating pure colloid)	This is an example of 4 specimens examined. All have approximately the same thyroid condition. Colloid dense, nearly uniform. Follicles more separate and stroma more prominent, due to shrinkage of blood supply and size of follicles. Epithelium cuboidal.

[3] Complete examinations of all the fry at the hatchery were made in August, and all were found to have thyroid hyperplasia (Marine D., *Jour. Exper. Med.*, 1914, xix, 70).

Pond No.	Age in months.	Date taken.	Food and length of time fed.	Condition of thyroid.		Remarks.
				Extent of overgrowth.	Classification.	
8	9+	Nov. 2, 1913	Butter fish only for 5 wks.	Entire subpharyngeal area filled, extending to pharyngeal mucosa, to bone, muscle, and gills	Colloid goitre (complete involution)	This is an example of 4 specimens examined. All have approximately the same thyroid condition. Colloid dense, uniform. Follicles separate. Epithelium low cuboidal.
8	9+	Nov. 9, 1913	Butter fish only for 6 wks.	Entire subpharyngeal area filled, extending to pharynx mucosa, bone, and muscle	Colloid goitre (complete involution)	This is an example of 4 specimens examined, all showing same thyroid condition.
8	10	Nov. 16, 1913	Butter fish only for 7 wks.	Entire subpharyngeal area filled, extending to skin, pharyngeal mucosa, bone, and muscle	Colloid goitre (complete involution)	This is an example of 4 specimens examined, all showing same thyroid condition.
8	10+	Nov. 23, 1913	Butter fish only for 8 wks.	Entire subpharyngeal area filled, in bone and muscle	Colloid goitre (complete involution)	This is an example of 4 specimens examined, all showing same thyroid condition.
8	10+	Nov. 30, 1913	Butter fish only for 9 wks.	Entire thyroid area filled, extending to pharyngeal mucosa, to bone, and muscle	Colloid goitre (complete involution)	This is an example of 4 specimens, all showing same thyroid condition.

are still present at the colloid-epithelium junction. By the use of less rigid standards than I have adopted, these thyroids could be classified as completely involuted rather than as "colloid-early hyperplasias." By comparing the follicles with those of normal fish or with those seen in the subsequent specimens of this series, however, it is evident that the involution is not complete. The specimens taken after thirty-five days of fish feeding are listed as "complete involutions"; that is, the lining epithelium is low cuboidal. The stainable colloid is homogenous, and nearly uniform in all follicles. The follicles are smaller and more widely separated from each other, while the stroma is correspondingly more prominent. The capillaries about the follicles are less prominent. The distribution of the follicles is, of course, the same as that attained during the growing actively hyperplastic stage, *i. e.,* in bone and muscle with extension to the skin and pharyngeal mucosa.

The specimens examined on the 42d, 49th, 56th, and 63d days respectively are nearly identical with those of the 35th day. There is probably some further involution in the last specimens, as evidenced by a slight increase in the staining density of the colloid and in the flattening of the lining epithelium. This is also true of normal thyroid follicles, and has occasioned much discussion as to what is the normal type. In a tissue like the thyroid epithelium, which is capable of such marked hypertrophy and hyperplasia, as well as marked involution, one must adopt somewhat arbitrary groups, within which variations between certain narrow limits are not taken into account.

The time in which complete involution of these relatively mild degrees of thyroid hyperplasia occurs, following the use of butter fish in this particular environment and strain of brook trout, is about thirty-five days. As was shown in a previous paper (1), the thyroid gland, once involuted, remains in this colloid or involuted stage as long as the fish are fed with this food. No further growth of the thyroid takes place under these conditions. Thus the thyroids of the three and two year old brook trout at this hatchery have the same amount of thyroid tissue with the same distribution of follicles that was attained during the first ten months of their lives, at which time the change of food was instituted.

Iodin involutes the hyperplasias more rapidly than butter fish. In Lake Erie pike with mild degrees of thyroid hyperplasia it was found (2) that iodin involuted them in from sixteen to eighteen days.

In the experiments of 1909 (3) it was found that the thyroid of twenty months old brook trout with extensive hyperplasia involuted in from twenty to twenty-five days following the daily addition of traces of iodin to the water. In the iodin experiments of 1910 (4) it was found that involution took place in about forty days in twenty-nine months old brook trout with large and often slightly ulcerated goitres, while in forty-one months old emaciated brook trout with extensively infected and ulcerated external goitres healing and involution were complete at the sixty-second day. In mammals the involution with iodin is of course more rapid (5). In young dogs with uncomplicated simple hyperplasias, the involution is usually complete in from fourteen to eighteen days.

As the whole butter fish contains appreciable amounts of iodin[4] one may ask whether this effect of fish as food is merely the well known iodin action, and the somewhat longer involution time only the manifestation of a very dilute solution. In the absence of any data against this view, I prefer tentatively to consider it an iodin effect rather than to postulate another explanation in favor of which there are at present no direct data. Experiments are now in progress which it is hoped will shed some light on this question.

[4] Iodin in fish, birds, and mammals is, for the most part, contained in the thyroid glands, although traces of iodin have been found in certain fish oils, as, for example, cod liver oil, where presumably contamination with thyroid was avoided. Using the modified Baumann and the Hunter methods, I have not been able to recognize traces of iodin in whole amphioxus and in butter fish (*Stromateus triacanthus*) and weak fish (*Cynoscion regalis*) from which the thyroid areas had been removed. A. T. Cameron (*Biochem. Jour.*, 1913, vii, 466; *Jour. Biol. Chem.*, 1914, xvi, 465) has published the finding of relatively large amounts of iodin in the thyroids of rays and dog fish. Thus in *Raia clavata* he found the mean iodin content to be 0.438 per cent. of dried gland, while in *Scyllium canicula* he found a mean content of 1.16 per cent. of dried gland. I have made the following iodin estimations in butter fish:

(1) With whole butter fish in 1 gm. amounts = possible trace.
(2) With whole butter fish in 5 gm. amounts = trace, unmeasurable.
(3) With butter fish, exclusive of the thyroid area, in 1 gm amounts = no trace.
(4) With butter fish, exclusive of the thyroid area, in 5 gm. amounts = no trace.

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A STUDY OF THE FERMENTS AND FERMENT-INHIBITING SUBSTANCES IN TUBERCULOUS CASEOUS MATERIAL.

Studies on Ferment Action. XII.*

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In a previous paper (1) we reported that sodium soaps prepared from ether-soluble extractives of tubercle bacilli inhibit the action of trypsin and leucoprotease. The inhibiting action was found to depend on the presence of soaps of the unsaturated fatty acids, and could be destroyed by saturating the acids with iodin.

The results of this study appear to have a direct bearing on the development of caseation in tuberculosis. Caseation in tuberculosis is a form of coagulation necrosis in which the dead tissues rarely undergo autolysis, except as a result of secondary infection. Syphilis is the only infectious disease presenting a similar condition. In other instances of coagulation necrosis the dead tissues are soon removed by means of autolysis and phagocytosis. It appears, then, that substances having the property of preventing autolysis must be present in syphilitic and tuberculous tissues.

This view is confirmed by the work of Schmoll (2) who was unable to find any evidences of autolysis in caseous material. The results obtained by Auclair (3) can be explained in a similar manner. Auclair believes that caseation is due to specific toxins of the nature of fats which are soluble in ether, chloroform, and benzin. These extracts suspended in water produced typical caseous abscesses when injected into the subcutaneous tissues of animals, and caseous areas in the lungs when injected into the trachea of guinea pigs. No doubt the conditions observed by Auclair closely simulated caseous areas, but we doubt if they were due to the presence of specific toxins. It is more probable that his injections caused the usual inflammatory exudate which assumed the character of the caseous matter owing to the failure of autolysis.

The lack of autolysis in caseous material is similar to that observed in non-

* Received for publication, January 21, 1914.

infected anemic infarcts. In the latter condition the dead tissue is finally removed by the phagocytic leucocytes which invade it from the surrounding tissues. The leucocytes also liberate ferments which aid in causing solution and absorption of the dead tissue. It is only in the large infarcts that softening occurs, and this is in the central part of the mass, while the periphery remains firm. Wiener (4) found that the autolytic enzymes act best in an acid medium, and Wells (5) believes that this explains the central softening in large infarcts. According to Wells, the acids at the periphery are neutralized by the blood plasma, so that autolysis is active only at the center.

Anemia, due to occlusion of the blood vessels, may be an important factor in causing caseation in the chronic forms of tuberculosis. However, in acute caseous pneumonia some other explanation seems necessary. Here we do not have the connective tissue reaction seen in the chronic forms of the disease, and we must therefore look for some other cause to explain the conditions present.

In a previous report (6) we showed that tubercle bacilli contain unsaturated fatty acids which, when saponified, are able to inhibit enzyme action. We believe that this observation explains the lack of autolysis in caseous matter, but it was considered necessary to make a careful study of caseous material in order to find out if ferments and ferment-inhibiting substances were present.

Schmoll's (7) failure to find evidences of autolysis in the caseous material from lymph glands indicates that ferment action has been inhibited from the start. This assumption is based not only upon Schmoll's work, but also upon our observation (8) that trypsin is destroyed when incubated for thirty minutes with soaps of the unsaturated fatty acids. If this view is correct, failure of proteolysis is due to destruction of the ferments by the soaps, but we must also consider the possibility of undestroyed ferments being present, though inactive, owing to the presence of inhibiting substances. It is unlikely that free ferments should be present in the caseous material obtained from chronic tuberculous lymph glands, but they may be present in a rapidly progressing caseous pneumonia with a marked inflammatory exudate. In the latter condition it is impossible to separate the caseous material from the inflammatory exudate, and so extracts of the caseous material will probably show the presence of some undestroyed ferments.

The caseous matter used in this study was obtained from tuberculous lymph glands and from several cases of rapidly progressing

caseous pneumonia.[1] The method used to determine the extent of digestion was that described in our previous reports. The mixture was made definitely acid with a solution composed of 10 per cent. acetic acid and 20 per cent. sodium chloride. The tubes were then placed in boiling water for five to ten minutes to coagulate the protein and the contents afterwards filtered through kaolin. The total incoagulable nitrogen was determined according to the method recommended by Folin.

CASEOUS LYMPH GLANDS.

The caseous matter derived from the lymph glands was spread in thin layers on glass plates and dried at temperatures below 45° C. After being dried the material was ground in a mortar to a fine powder. For the experimental work the emulsions were prepared in the proportions of one gram of caseous matter to 100 cubic centimeters of water. The material was examined to find out if ferments and ferment-inhibiting substances were present and also for evidences of autolysis. To determine the extent of autolysis a flask containing fifty cubic centimeters of the emulsion was placed in the incubator and left there for five days. Every day five cubic centimeters were removed and the amount of incoagulable nitrogen was determined. The incoagulable nitrogen obtained in five cubic centimeters of the emulsion on the fifth day was practically the same as that obtained on the first day. The results of this experiment indicate that either ferments are not present in the caseous matter, or their activity is suppressed. The freshly prepared emulsion was neutral in reaction, but became slightly acid after the second day. This is important as in the beginning the reaction was favorable to the ferments active in an alkaline or neutral reaction, and after the second day it was favorable to those active in an acid reaction, yet autolysis did not occur.

We obtained somewhat different results in a similar experiment conducted with caseous material from a lymph gland which had become secondarily infected. Autolysis occurred in a more marked

[1] We wish to thank Dr. F. Mathews of St. Mary's Free Hospital, Dr. Larkin of the New York Hospital, and Dr. Lamb of the Presbyterian Hospital for their kindness in furnishing us with material.

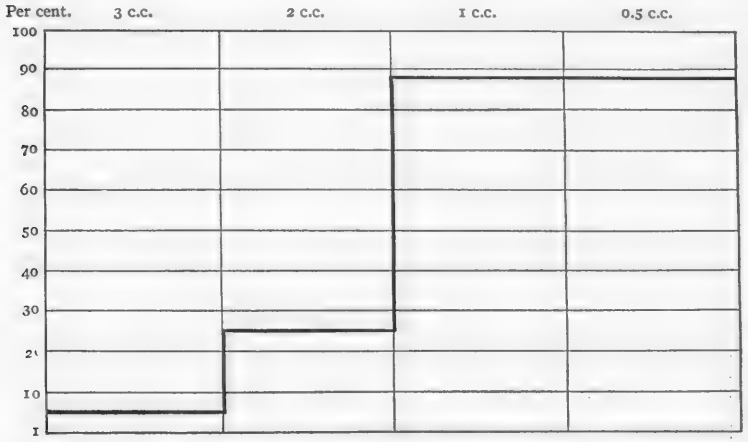
degree in an alkaline reaction, but was much more active in an acid reaction. In no instance have we been able to obtain evidences of autolysis in emulsions of uninfected caseous matter of lymph glands, either in acid or alkaline reactions. The lack of autolysis in the caseous material might have been due to the presence of soaps, and we therefore attempted to rule out this factor.

Two methods were used to determine the presence of undestroyed ferments. In the first the whole emulsion was treated with an excess of alcohol to which a small amount of hydrochloric acid had been added. Treated in this manner the ferments and protein were precipitated, and the fatty acids liberated by the hydrochloric acid were made soluble in the acid alcohol. The precipitate was then washed with alcohol and ether, and dried. The powder thus obtained was made into an emulsion with water, a portion made acid and another alkaline, and placed in the incubator for several days. The presence of ferments would have been shown by the degree of autolysis that occurred. In the second method the emulsion was first incubated for twenty-four hours in an alkaline solution in order to get the ferments and soap into solution. It was then centrifuged and the supernatant fluid treated as was the whole emulsion. In this instance the powder was made into an emulsion and tested against casein. In neither case were we able to demonstrate the presence of ferments.

We believe that the results of these experiments justify us in concluding that undestroyed ferments are rarely present in caseous lymph glands which have not become secondarily infected.

As practically all cells contain intracellular ferments, we must assume that substances were present which prevented autolysis. Our previous work indicated that these substances were probably soaps, therefore our next experiments were planned to see if this was the case. A fresh 1 per cent. emulsion was prepared and kept at room temperature for several hours. During this period it was shaken frequently. It was then centrifuged until it became clear. The supernatant fluid was now removed, made strongly alkaline with sodium hydrate, and the flask containing it placed in boiling water for several minutes. The fatty acids were then liberated with hydrochloric acid, taken up in ether, and resaponified.

The last step was repeated in order to free the soaps from unsaponifiable matter. The soaps were then tested for their enzyme-inhibiting action. They were mixed in various dilutions with trypsin and placed in the incubator at 37° C. for thirty minutes. The casein was then added and the mixture placed in the incubator again for about two hours. After the final incubation the total incoagulable nitrogen was determined according to Folin's method. Text-



TEXT-FIG. 1. Effect of the saponified extract from caseous lymph glands on tryptic digestion.

figure 1 shows the influence of soaps obtained from the caseous matter of lymph glands on the activity of trypsin.

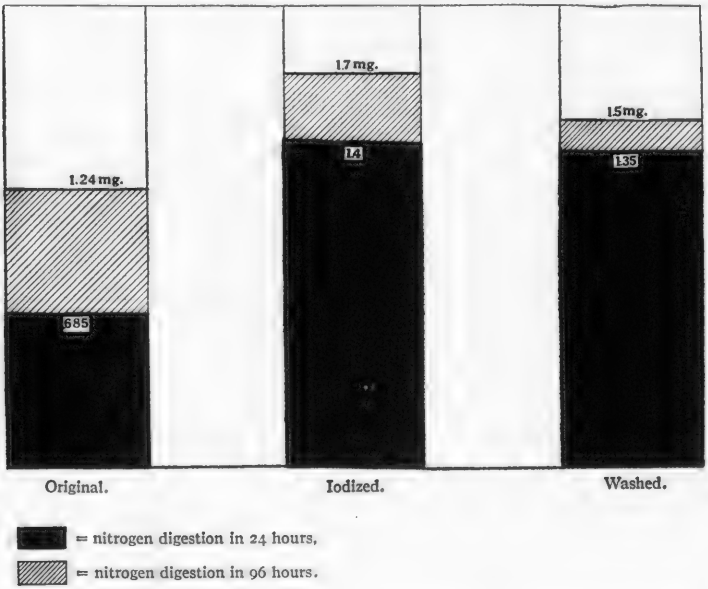
The soaps had been dissolved in sufficient water so that one cubic centimeter of the solution represented the amount of soap contained in one cubic centimeter of the original 1 per cent. emulsion. Three cubic centimeters of the emulsion, representing the soap contained in 0.03 of a gram of caseous matter, was sufficient to inhibit the action of the ferment. In the tube containing two cubic centimeters the digestion was only 25 per cent. of that obtained in the control tube containing no soaps.

The results obtained with the caseous material taken from a lymph gland which had become secondarily infected differ from those just described. Similar experiments were conducted with

this material, which was semifluid in consistence, and ferments active in alkaline and acid reactions were demonstrated. While autolysis occurred in both acid and alkaline reactions, it was more marked in the acid reaction, indicating that soaps were still present.

With this possibility in mind we decided to study the influence of iodin on the whole emulsion to see if it would accelerate autolysis. We have already shown (9) that treating the soaps of the unsaturated fatty acids with iodin destroys their ferment-inhibiting action, and it is therefore of interest to determine if similar results can be obtained with the native mixtures of protein and soaps.

The emulsion was divided into two portions, one of which was used as a control. To the other was added a mixture of iodin and iodide of potassium. The mixture was allowed to stand for several hours, and the excess of iodin was then removed by repeated extractions with chloroform. A third flask containing the washed precipitate was also used. To each of these flasks trypsin was



TEXT-FIG. 2. Effect of iodin on ferment-inhibiting substances present in caseous matter of lymph glands.

added. Text-figure 2 shows the amount of incoagulable nitrogen in two cubic centimeters of the emulsion after twenty-four and ninety-six hours' incubation at 37° C. The first column shows the amount of incoagulable nitrogen obtained after digesting the untreated emulsion with trypsin. The second column represents the amount of nitrogen obtained by digesting the iodized fraction, and the third the action of trypsin on the precipitate which had been freed from soaps by extraction with water and repeated washings.

A study of this chart shows that in the first tube soaps were not present in sufficient quantity to inhibit the action of the trypsin, but that digestion in a similar fraction treated with iodin was as great at the end of twenty-four hours as that obtained in the first tube after ninety-six hours. In the third tube the soaps had evidently been removed to a large extent, though digestion in the last seventy-two hours was not equal to that observed in the iodized fraction.

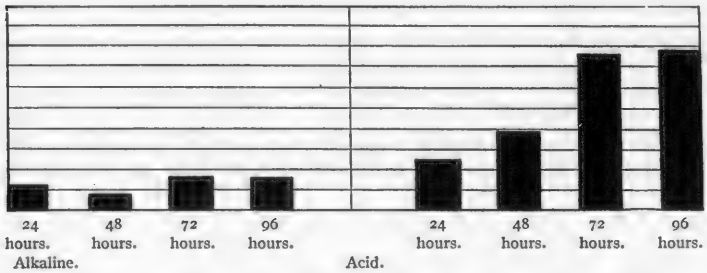
CASEOUS PNEUMONIA.

The affected areas in the caseous lungs were freed as much as possible from the less involved lung tissue, cut up in a meat machine, and dried. When dry the material was ground to a fine powder in a mortar. We anticipated somewhat different results in these experiments from those obtained with caseous material from lymph glands, owing to the difficulty of freeing the caseous material from the inflammatory exudate. In the former case there is usually no inflammatory exudate and therefore no extraneous ferments, while in the material from caseous pneumonia these ferments must be considered.

Several specimens of this kind were examined, and practically similar results were obtained with all. As in the experiments conducted with the caseous matter of lymph glands, the emulsion was made in the proportion of one gram of the dried material to one hundred cubic centimeters of water.

In the first experiment we wished to determine the extent of autolysis. Two flasks containing twenty-five cubic centimeters each of the emulsion were incubated for several days at 37° C. In order to determine the type of ferments present, the contents of one of

the flasks was made slightly acid in reaction, and the other slightly alkaline. Text-figure 3 shows the result of this experiment. In studying this chart it should be remembered that soaps are dissociated in an acid reaction, and are therefore inactive as inhibiting agents. Each line on the chart represents 0.1 of a milligram of nitrogen, and the black columns indicate the incoagulable nitrogen in



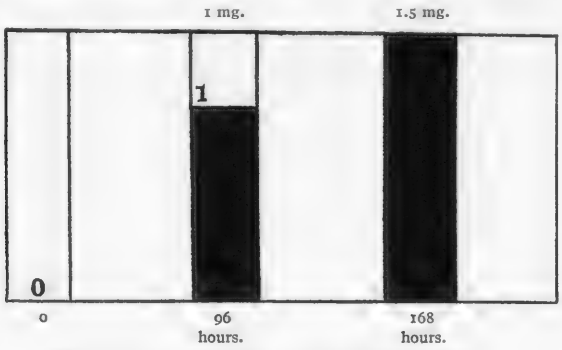
TEXT-FIG. 3. Autolysis of caseous material in acid and alkaline reactions.

two cubic centimeters of the emulsion. The chart shows the differences obtained in the presence of acids and alkalies. Inhibiting substances were present in sufficient quantity to prevent the action of ferments active in an alkaline reaction, but they were not effective when the reaction was made acid.

In some of our preliminary experiments we observed that autolysis occurred in certain of the emulsions which had been made slightly alkaline, and we were at a loss for the proper explanation as soaps were known to be present. We found later that in practically all such instances the reaction of the emulsion had become acid, thus throwing out the soaps and permitting the action of ferments active in an acid reaction. In the last experiment it was evident that ferments active in an acid reaction were present, but that those active in an alkaline reaction were absent or their activity was suppressed by the soaps. In the next experiment we wished to determine if ferments active in an alkaline reaction were also present in the caseous material.

The emulsion was treated with an excess of alcohol which had been made slightly acid. By this means we hoped to precipitate the ferments uninjured and at the same time to remove the soaps, or

rather the fatty acids, which, treated in this manner, are soluble in alcohol. The precipitate was removed by centrifugalization, washed with alcohol and ether, and dried. It was then brought up to the original volume with water, made slightly alkaline, and placed in the incubator for seven days. The results of this experiment are seen in text-figure 4. The columns represent the amount of incoag-



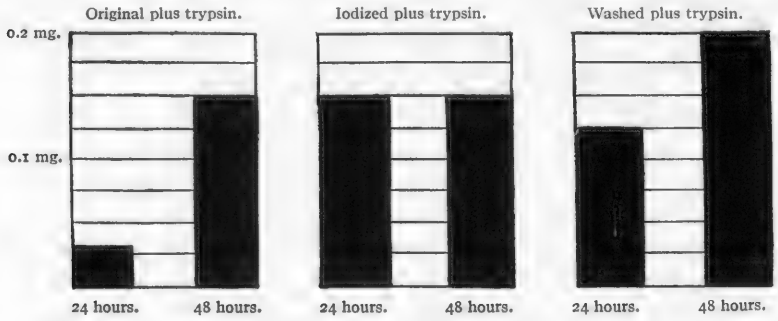
TEXT-FIG. 4. Autolysis of alcohol precipitate.

ulable nitrogen in two cubic centimeters of the mixture. The chart shows that ferments active in an alkaline reaction were present in the original material, but that their activity was inhibited by the soaps. When these were removed by the acid alcohol, the caseous matter underwent autolysis. It is unlikely that these tissues become acid during life, and so the lack of autolysis is easily explained.

Soaps were present in smaller amounts than in the caseous matter of lymph glands. This may be due to the fact that the rapidity of the process did not permit an accumulation of the soaps such as probably occurs in the chronic tuberculous lymph glands. Text-figure 3 shows that autolysis did not occur when the original emulsion was made slightly alkaline, though in other experiments in which the soaps were first removed, we demonstrated the presence of ferments active in an alkaline reaction. These results indicate a slight excess of soaps over the amount needed to prevent autolysis. This view is confirmed in the next experiment in which it is shown that with an increase of ferment autolysis ensues.

In our experiments with the caseous matter of lymph glands we

showed that previous treatment of the emulsion with iodin was effective in aiding the action of trypsin. We now made similar experiments with the pneumonic material, using the same technique. Text-figure 5 shows the results obtained. Two controls were used.



TEXT-FIG. 5. Effect of iodin on ferment-inhibiting substances in caseous pneumonia.

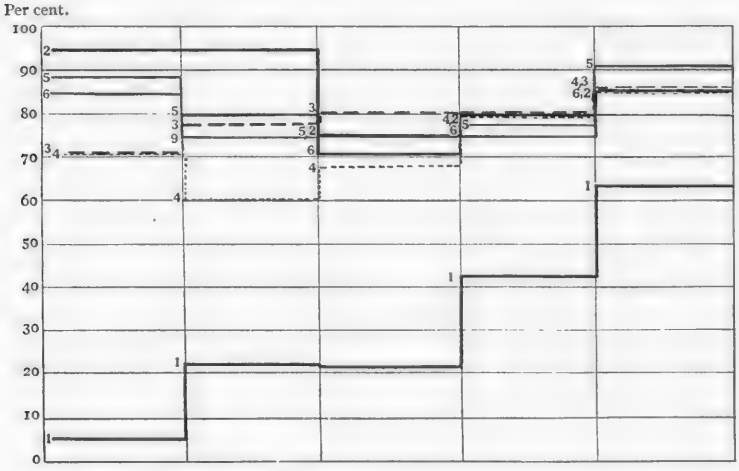
One control consisted of the untreated emulsion with trypsin, and the other of the washed precipitate with trypsin. This last control was used to see if all the soaps could be removed from the precipitate by repeated washings with water.

Here again we have evidence that previous treatment of the emulsion with iodin increased the activity of the trypsin. The amount of incoagulable nitrogen obtained after twenty-four hours' incubation of the iodized emulsion was much greater than that observed in the untreated fraction, and also greater than that obtained with the washed precipitate. It is probable that the washed precipitate was not entirely free of soaps. The amount of digestion observed after forty-eight hours in the untreated emulsion indicates that soaps were not present in large amounts. In a previous paper (9) we showed that soaps were able to inhibit completely the action of enzymes only in certain proportions, and that digestion occurred when enzymes were present in excess of this proportion. This is probably the explanation in the present instance, as the results show that soaps are present in comparatively small amounts in these pneumonic areas (text-figure 6).

In addition to the extractives of the tubercle bacilli which we have

been discussing, lipoids derived from dead cells are also present in caseous matter. Neumann (10) and others state that an alkaline solution of lecithin inhibits tryptic activity, but Meyer (11), using a 1 per cent. solution, was unable to confirm these observations.

In view of the uncertainty of the action of the lipoids we made a few experiments in order to determine their influence on enzyme activity. For these experiments lecithin, cuorin, and cholesterol were used. The solutions were prepared by mixing one gram of the lipoid with 100 cubic centimeters of a 1 per cent. solution of an inactive preparation of sodium oleate. This sodium oleate prepara-



1 = soaps of tubercle bacilli.
2 = sodium oleate (saturated).
3 = soaps of tubercle bacilli (from waxes).
4 = lecithin.
5 = cuorin.
6 = cholesterol.

TEXT-FIG. 6. Effect of soaps of tubercle bacilli and lipoids on tryptic digestion.

tion was one in which the acid had become saturated, thereby rendering it inactive as an inhibiting agent. By suspending the lipoids in an inactive soap solution we hoped to duplicate the physical condition present in the control containing the soap prepared from tubercle bacilli. Text-figure 6 gives the results obtained in one of the experiments.

In addition to the preparations mentioned above, there was also tested in this experiment a soap prepared by saponifying the acetone-insoluble fraction of the extractives of the tubercle bacilli. This soap is apparently much less active than that prepared from the acetone-soluble fraction.

All the lipoids when tested in this manner appear to have some inhibiting action, particularly lecithin which is the most active. The experiment indicates that lipoids in the tissues, particularly in the presence of soaps, may aid in preventing autolysis, but their activity is much less than that demonstrated for the soaps.

DISCUSSION.

We believe that we are now in a position to understand more clearly the processes leading to caseation in tuberculosis. With the lodging of the bacilli there is death of some of the cells due to the action of the toxins, and a proliferation of the neighboring cells. Under other conditions the death of a small number of cells would be followed at once by their removal, either through the action of phagocytes or by autolysis; but here the phagocytes are absent, and the action of the enzymes is prevented by the presence of the soaps of the unsaturated fatty acids derived from the tubercle bacilli. The anemia caused by the occlusion of the blood vessels plays an important part, as the lack of fluid prevents the dilution and subsequent absorption of the inhibiting substances. As the local process spreads slowly, the reaction at the periphery of the area prevents the entrance of fluids, and thus the caseous area gradually increases in size as the bacilli invade and cause the death of the surrounding tissues.

In conditions such as those present in caseous pneumonia, there is primarily an exudate composed of desquamated cells, leucocytes, and fibrin, which soon becomes caseous. Before caseation is complete in the zone of gelatinous pneumonia, there is not the anemia due to occlusion of vessels that is seen in the more chronic processes, and we must conclude that persistence of caseation is due to substances inhibiting the action of the ferments present in the inflammatory exudate. An important factor in the acute process under discussion is the immense number of bacilli present. Under these conditions

it is not difficult to imagine a corresponding increase in the amount of inhibiting substances, an increase sufficient to neutralize the ferments liberated by the disintegration of leucocytes and other cells. Caseous areas may persist for a long time without undergoing absorption. The rapid softening of these areas subsequent to secondary infections is probably due not only to the entrance of fresh ferments, but also to the fluid accompanying these conditions, the fluid acting as a diluent and aiding in the removal of the inhibiting substances. Soaps if present must exist in very minute quantities in the bacilli. They are probably formed from the neutral fat of the bacilli. The lipases hydrolyze the fats, and the acids are then saponified. Klotz (12) and Bergell (13) have demonstrated the presence of lipases in tuberculous pus and lymphocytes, while Wells and Corper (14) found the ferment present in the tubercle bacilli.

According to Sata (15), most of the fat present in caseous matter is found at the periphery of the area. This may be due either to a wandering in from the outside, or, more probably, to synthesis from the fatty acids liberated during the disintegration of the cells and of the tubercle bacilli. Our experiments indicate that soaps of the unsaturated fatty acids obtained from tubercle bacilli are the active agents in preventing autolysis, and the identification of these acids will probably aid in determining the origin of the neutral fat found in caseous areas.

In our paper on the ferment-inhibiting substances present in tubercle bacilli (16) we reported that iodin combined with the unsaturated fatty acids obtained from the bacilli and neutralized their ferment-inhibiting properties. We also emphasized the importance of this observation in connection with a specific chemotherapeutic agent for tuberculosis. In our present work we have shown that iodin exerts a similar action on the ferment-inhibiting substances present in caseous matter. Thus the iodin may serve another purpose by aiding in bringing about solution and absorption of the caseous matter, and exposing the bacilli, which otherwise are more or less inaccessible, to the influence of the therapeutic agent.

It is a well known clinical observation that iodides cause tubercle bacilli to appear in the sputum of patients with pulmonary tuberculosis, though they were previously absent. This phenomenon is

probably due to the neutralization of the action of the ferment-inhibiting substances by the iodin. Subsequent to this neutralization, ferment action ensues, with liberation of the bacilli and their appearance in the sputum.

The autolysis that occurs in the center of large anemic infarcts, and the lack of autolysis at the periphery, can be explained in the same manner. At the periphery of the infarct the fatty acids which are liberated following the death of the cells are saponified by the alkalies present in the tissues, and by those coming from the surrounding fluids. The soaps thus formed inhibit proteolysis until the phagocytes, invading the periphery, open up channels through which sufficient fluid can enter to wash out or dilute the soaps so that autolysis can proceed. The phagocytes of course also take part in the removal of the dead tissues. In the center of the infarct the alkalies are soon used up and the tissues then become acid in reaction on account of the excess of fatty acids. Wiener (17) found that autolytic enzymes act best in an acid medium, and this is to be expected as soaps are inactive as inhibiting agents under these conditions.

CONCLUSIONS.

1. Caseous matter obtained from lymph glands which have not become secondarily infected contains substances which inhibit enzyme activity. These substances consist chiefly of soaps of the unsaturated fatty acids.

2. The inhibiting substances are present in relatively smaller amounts when the caseous matter has become secondarily infected. This is probably due to the dilution and washing out of the soaps.

3. Ferments are either entirely absent or present in very small amounts, unless the caseous matter has become secondarily infected.

4. Caseous material from the lungs contains smaller amounts of the inhibiting substances. This may be due to the acuteness of the process, which does not permit an accumulation of the soaps, or to the binding of the soaps with the ferments.

5. Ferments are present in caseous pneumonia. In the whole emulsion the ferments are less active in an alkaline than in an acid reaction; but removal of the soaps shows that those active in an alkaline reaction are also present in considerable amounts.

6. The previous treatment with iodin of caseous matter from both lymph glands and lungs increases the action of the trypsin.

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THE EFFECT OF DILUTION OF PLASMA MEDIUM ON THE GROWTH AND FAT ACCUMULATION OF CELLS IN TISSUE CULTURES.*

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PLATES 43 TO 45.

Since the method of cultivating tissues *in vitro* will no doubt be used in investigating the action of various substances on the growth and function of cells, it is important that the influence on cells, growing under these conditions, of certain easily modified physical and mechanical factors should be understood. The importance of these factors is indicated by the fact that so simple a matter as the thickness of the hanging drop of plasma influences markedly the extent of the outgrowth of cells from the original fragment of tissue.

In a former paper[1] we discussed the influence of temperature, including the effect of variations in the temperature of incubation as well as the effects of exposure of the cultures to high and low temperatures before incubation. In the present paper we shall consider the influence of another physical factor, namely dilution of the plasma medium, on the behavior of cells in cultures. Carrel and Burrows[2] (1911) were the first to describe the effect of dilution with distilled water on cell wandering, and the subject was also discussed in a recent paper by Burrows[3] (1913). Some of the work reported in this paper was begun soon after the appearance of the experiments of Carrel and Burrows, with the object of testing the correctness of the interpretation of their results regarding the

* Received for publication, January 24, 1914.

[1] Lambert, R. A., *Anat. Rec.*, 1912, vi, 91.

[2] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 562.

[3] Burrows, M. T., *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 77.

stimulating action of hypotonic plasma. Later, the use of the method of dilution for the study of the accumulation of fat by cells *in vitro* was suggested to us, and further experiments with this aim in view were carried out. We shall report here the results of two sets of experiments, (1) upon the effect of dilution on the migration and multiplication of cells in cultures, and (2) upon the effect of dilution on the accumulation of fat by cells in cultures.

THE EFFECT OF DILUTION OF PLASMA ON THE MIGRATION AND MULTIPLICATION OF CELLS.

Carrel and Burrows[4] reported that the addition of a limited quantity of water to chick plasma caused a more extensive outwandering of cells in cultures of chick embryo spleen. They also observed that the addition of hypertonic salt solutions was harmful. The stimulating effect in the first instance was attributed to the hypotonic state of the medium. No experiments with isotonic solutions as diluents were made. Burrows has recently published the results of studies upon the effect of the addition of such solutions. A summary of his work is given below in connection with the results that we have obtained.

We have repeated the experiments of Carrel and Burrows with hypotonic plasma, preparing at the same time cultures in plasma diluted with serum and with Ringer's solution. In addition to chick embryo spleen, we have used for cultivation chick embryo heart, bone marrow and intestine, rat embryo skin, and mouse carcinoma.

The Effect of Dilution on the Migration of Cells.—The first experiments consisted in the preparation of cultures of chick embryo spleen in the following media: (1) undiluted plasma, (2) plasma diluted with distilled water in the proportion of 3 to 2 (the optimum medium of Carrel and Burrows), (3) plasma similarly diluted with Ringer's solution, and (4) with serum, and (5) plasma diluted with serum in the proportion of 1 to 2.

Care was taken to have the pieces of tissue of uniform size, and the drops of plasma of as nearly uniform thickness as possible. Ten preparations were made with each medium. At the end of forty-eight hours' incubation, after which time experience had

[4] Carrel, A., and Burrows, M. T., *loc. cit.*

shown that little further migration of spleen cells took place, the cover-glasses with the clots attached were placed in formalin and later mounted on slides. With a projection drawing apparatus rough drawings were made in which the pieces of tissue and the halos of migrated cells were outlined. By taking the average of the radii of the tissue fragments and the average width of the cell halos, a composite drawing for each series of preparations was constructed. While the cultures in a single series differed little from one another, the composite drawings of the several series showed certain differences (figures 1, 2, 3, 4, and 5). The most extensive migration is seen to have taken place in the plasma diluted with two volumes of serum. There is little difference in the extent of migration about the pieces of tissue in plasma diluted with the smaller quantities of serum, Ringer's solution, and water, although in each the outwandering was more extensive than in the controls in undiluted plasma. In other words, the most active migration took place in the plasma diluted with the largest quantity of fluid, and not in the medium made hypotonic by the addition of distilled water. This result is in agreement with the recent work of Burrows in which he found that the extent of migration of spleen cells in plasma diluted with varying quantities of serum increases with the amount of serum added. The effect is attributed to the diminution in the amount of fibrin in the clots. Since this reduction in fibrin results, in the beginning at least, in a coarser meshwork in the clot, the explanation of the increased migration of cells would seem to lie in the diminished resistance to the progressive movements of the cells. In order to test this explanation several other tissues were used, including chick embryo heart, intestine and bone marrow, rat embryo skin, and mouse carcinoma, the cells of which vary considerably in their migratory ability.

Briefly stated, it was found that cells which are relatively small and actively motile, such as those of the bone marrow and spleen, show increased migration in diluted plasma, whereas cells whose power of locomotion is limited, such as those of intestinal epithelium, rat embryo skin, and mouse carcinoma, which tend to spread out slowly in cultures in groups or large sheets, are not influenced by dilution of the medium. Cells which occupy a midway position

in the matter of motility, such as connective tissue cells, are only slightly influenced in this way. The mechanical explanation of diminished resistance to active cell locomotion offered by the coarser fibrin meshwork seems to us to account for these results.

The Effect of Dilution on Cell Multiplication.—An analysis of the results suggests other criteria for determining a true stimulating effect on tissue cultures than the extent of the outwandering of cells through the clot. It occurred to us that the extent of cell multiplication, as determined by the number of cells that undergo division in the preparations in a given time, would afford a much more satisfactory test. As we have pointed out, all stages of mitotic division can be observed with comparative ease in the living cell. Since division at body temperature is completed, as a rule, in from twenty to thirty minutes, the number of mitoses in a preparation may be hourly recorded without danger of counting the same cell twice. By making observations on small groups of preparations from the several series over a period of twenty-four hours or longer it is possible to arrive at a definite conclusion as to which series shows the most active multiplication of cells. We have studied cultures of chick embryo heart in pure plasma and in plasma diluted with water and with Ringer's solution in the proportions given above. There were five preparations in each set. Each preparation was examined six times during a period of forty-eight hours. The total number of mitoses for each of the three sets in the order stated was, 88, 96, and 81. In another experiment in which four observations were made the total numbers were, 76, 62, and 70. We concluded from these two experiments that dilution of plasma within these limits is without appreciable effect on cell multiplication.

THE EFFECT OF DILUTION ON THE ACCUMULATION OF FAT BY THE CELLS.

In previous communications we have referred to the appearance in the cells of tissue cultures of small droplets of fat which tend to increase in size and number with the age of the culture. After five to seven days the cells may be actually distended with the droplets.

We[5] reported observations which seemed to show that the accumulation was in all probability the result of a disturbance in cell metabolism. There was evidence that the vitality of the cells was not seriously impaired, since they were seen to move about actively and to undergo normal mitotic division. We also observed that cells growing in a thin drop of plasma accumulated less fat than those in a thick coagulum. This phenomenon can be easily demonstrated in a single preparation by shaking the drop of plasma before coagulation takes place, causing the drop to touch the slide cavity, and then by manipulating the tissue fragment so that it lies along the inner edge of the drop. The cells that grow in one direction into the thick clot accumulate a large quantity of fat in their cytoplasm, while those growing in the other direction in the thin film of medium on the cover-glass accumulate very little fat. It is difficult to say whether this difference in fat accumulation is due to the difference in the quantity of fat at the disposal of the cells, or to a difference in oxygen supply to the cells under the two conditions, as has been suggested by Burrows.

Several methods suggested themselves for testing the effect of a reduction in the quantity of fat in the medium on the amount of fat accumulated by the cells: (1) the removal of the fat by extraction with ether or chloroform; (2) the use of an artificial fat-free medium, such as agar or salt solutions, in which Lewis[6] states that embryonic chick cells will grow for a time; and (3) the use of plasma in which the amount of fat has been reduced by dilution with Ringer's or physiological salt solution. The first method was found impracticable because prolonged shaking of serum with pure ether or chloroform seemed to produce some change, probably in the serum proteins, as the result of which the serum even when combined with sufficient plasma to cause clotting did not afford a satisfactory culture medium. The second method could not be successfully used, because, after washing the tissue fragments in salt solution to remove the tissue lymph before preparing the cultures, it was found that the cells in the outgrowths lived only eighteen to twenty-

[5] Lambert, R. A., and Hanes, F. M., *Virchows Arch. f. path. Anat.*, 1913, ccxi, 100.

[6] Lewis, M. R., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 126.

four hours; that is, not long enough to make comparative observations. Very little fat is present in the pure plasma preparations at the end of twenty-four hours' incubation. We therefore resorted to the use of the third, or dilution method. Dilutions of chick plasma in Ringer's solution were made in the following proportions: 1 part of plasma to 2, 5, 10, 12, 15, and 20 parts of Ringer's solution. Clotting was not interfered with by the high dilution of the plasma, although the coagula formed by drops of the 1 to 15 and 1 to 20 dilutions were often not firm.

It was found that cells from pieces of chick embryo heart, previously washed in salt solution, showed active migration and multiplication in each of the diluted media. However, those growing in the 1 to 20 medium underwent early disintegration, frequently in less than twenty hours. The lower dilutions only were therefore used in the majority of the experiments. A single protocol will be sufficient to show the effect of dilution on the accumulation of fat by the cells; for it was found that when proper care was exercised in the preparation of the cultures, including the thorough washing of the pieces of tissue to prevent a relatively great modification of the dilution from the addition of tissue lymph, and in the care of the instruments and glassware used, the results in a number of experiments were practically uniform.

Experiment iii–5.—Plasma was obtained by bleeding a fowl from the wing vein. The heart of a ten day chick embryo was cut into small pieces of the size suitable for culture preparations. Dilutions of plasma were made by adding 1 part to 2, 5, 10, 12, and 15 parts of Ringer's solution. Ten pieces of tissue were put up in each medium, including undiluted plasma.

All preparations upon examination after twenty-four hours showed the usual radial extension of spindle and irregularly shaped cells. Small droplets of fat were visible in the cells in pure plasma and in those in the 1:2 and 1:5 dilutions; only occasional granules were seen in the preparations of the remaining series. After forty-eight hours the differences were more striking. There was a marked increase in the number and size of the granules in the cells in pure plasma and in the 1:2 dilution. In the 1:5 dilution the cells showed a moderate amount of fat, but decidedly less than those in undiluted plasma. Those growing in the higher dilutions were practically free from fat. Three of the preparations from each series were fixed in formalin at this time in order that the observations on the living cells could be verified by staining with Sudan III (figures 6 and 7). Some of the preparations in diluted media became disintegrated on the third and fourth days. Those remaining alive as long as four days presented striking pictures when compared with the controls, the former

showing only occasional granules of fat, while in the latter the cells appeared distended with numerous large droplets.

In order to exclude the possibility that in the diluted plasma the diminution in the amount of fibrin might have influenced in some way the fat metabolism of the cells, plasma diluted with serum instead of salt solution was used in another set of experiments. It was found that the accumulation of fat under these circumstances was practically the same as in undiluted plasma. Repetitions of these experiments, including several in which pigeon spleen and mouse carcinoma were used, gave results that varied little from those just stated. Two further observations, however, should be mentioned. First, it was occasionally observed that some of the cells that wandered farthest into the clot of highly diluted plasma showed a small number of rather large fat droplets, while the cells in the denser outgrowth immediately about the tissue fragment were practically fat-free; secondly, in a few of the preparations in diluted plasma in which the outgrowth consisted of only fifteen to twenty cells, these cells contained more fat than those in preparations showing a luxuriant outgrowth. These observations seem to be in harmony with the findings stated, for it may be assumed that if the number of cells in a culture or in a particular area of the clot is small the fat supply for these cells is relatively greater.

It has therefore been concluded that the effect of dilution of the plasma with salt solutions reduces the quantity of fat accumulated by the cells by reducing the quantity of fat in the medium.

SUMMARY.

1. Dilution of plasma with isotonic solutions causes a more extensive migration in cultures of cells of the actively migratory type, such as those of spleen and bone marrow. Dilution with a limited quantity of distilled water produces the same effect. Less actively motile cells are influenced little or not at all by dilution. The effect on cells of the first type is probably due to the reduction in the quantity of fibrin in the clot producing lessened resistance to cell locomotion.

2. Dilution of plasma with either isotonic solutions or distilled water is without effect on cell multiplication, as is shown by records of the number of mitoses in living culture preparations.

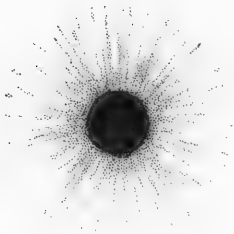


FIG. 1.

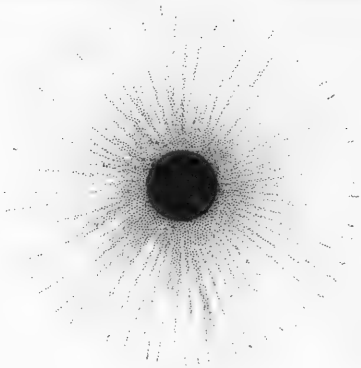


FIG. 2.

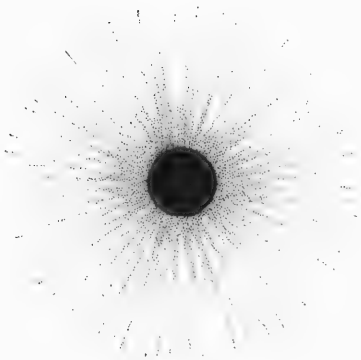
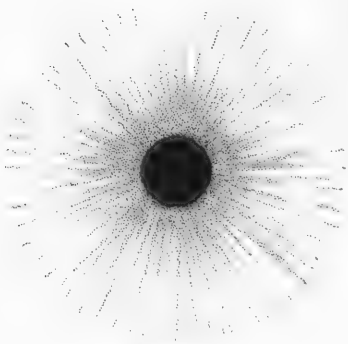


FIG. 3.



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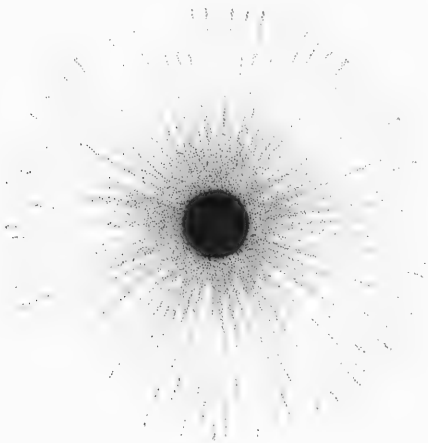

FIG. 5.

(Lambert: Growth of Cells in Tissue Cultures.)

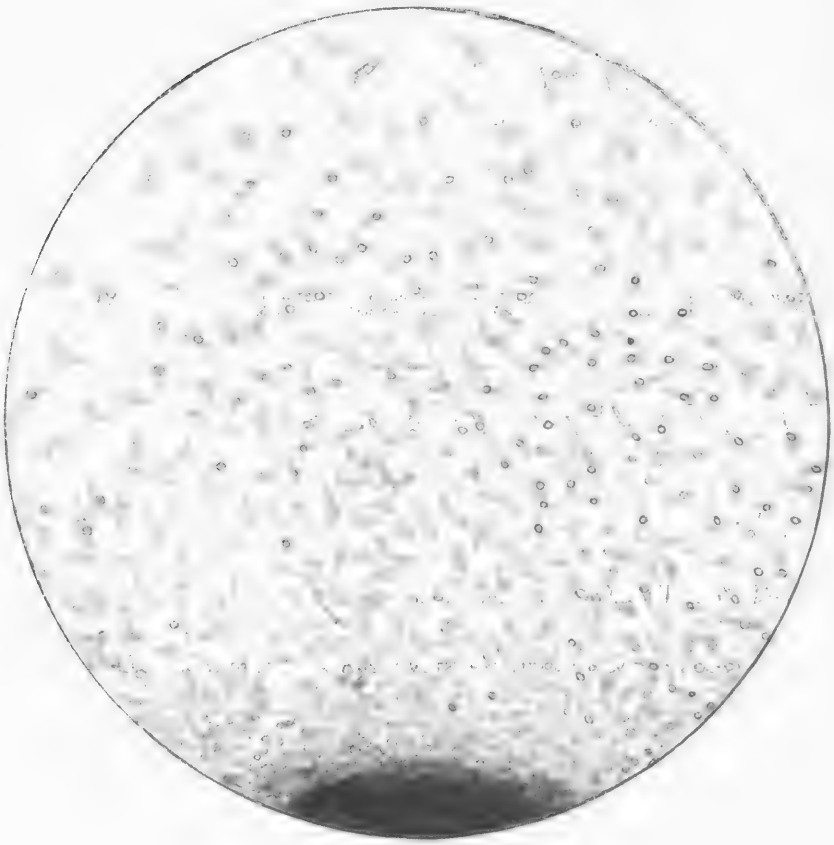

FIG. 6.

(Lambert: Growth of Cells in Tissue Cultures.)

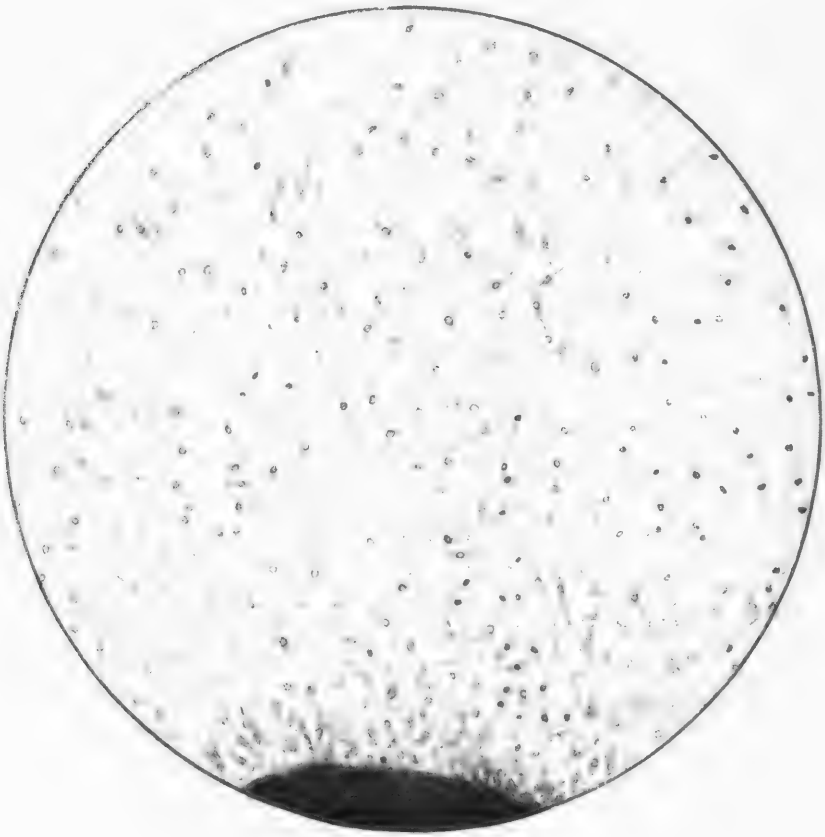

FIG. 7.

(Lambert: Growth of Cells in Tissue Cultures.)

3. Dilution of plasma with suitable quantities of Ringer's solution causes a marked diminution in the quantity of fat accumulated by the cells. This reduction is to be attributed to the decrease in the quantity of fat in the medium. The accumulation of fat by cells in cultures is therefore not to be regarded as the result of a cell degeneration, but as an accumulation, the source of the fat being the medium in which the cells are growing.

EXPLANATION OF PLATES.

Plate 43.

Drawings showing the extent of migration of spleen cells in undiluted chick plasma and in plasma diluted with serum and with salt solutions.

Fig. 1. In undiluted chick plasma (control).

Fig. 2. In plasma diluted with serum in the proportion of 3:2.

Fig. 3. In plasma diluted with distilled water in the same proportion.

Fig. 4. In plasma diluted with Ringer's solution in the same proportion.

Fig. 5. In plasma diluted with serum in the proportion of 1:2.

Plate 44.

Fig. 6. Three day culture of chick embryo heart in undiluted plasma. Stained with hematoxylin and Sudan III to show the accumulation of fat by the cells.

Plate 45.

Fig. 7. Three day culture of chick embryo heart in plasma diluted with Ringer's solution in the proportion of 1:15. The staining shows that fat granules are practically absent. It is also to be observed that the cells are smaller and stain somewhat more deeply with hematoxylin than in the control preparation.

A NOTE ON THE PATHOGENICITY OF TRYPANOSOMA LEWISI.*

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In the usual scheme of classification of trypanosomes, *Trypanosoma lewisi* occupies the position of the type species of a number of non-pathogenic trypanosomes.[1] While this usage is justifiable in the present state of our knowledge of these organisms, one must not lose sight of the fact that there is abundant evidence to show that *Trypanosoma lewisi* is not strictly non-pathogenic, but occasionally manifests a decided virulence for rats, especially young ones. Apart from such frequent disturbances as fever, anemia, and loss of weight, a considerable mortality may occur among infected rats. Perhaps the best instance that can be cited is that reported by Jürgens[2] who noted a mortality of 29.3 per cent. (16 out of 47) among young rats. Other authors have noted a slight mortality, or no mortality, resulting from infections of *Trypanosoma lewisi*. These differences in pathogenicity indicate that there are strains that differ fundamentally as regards their virulence. Delanoë[3] has added support to this conception of pathogenic and non-pathogenic strains of *Trypanosoma lewisi* by showing that while certain strains, or organisms from certain sources, are incapable of infecting mice, other strains may infect even a considerable percentage (40 per cent.) of the mice inoculated. Further, Roudsky[4] has shown by his "reinforced virus" that the virulence of a given strain is not absolutely fixed, but that it can be markedly increased for both rats and mice. Finally, Wendelstadt and Fellmer[5] have succeeded in

* Received for publication, February 15, 1914.

[1] Laveran, C. L. A., and Mesnil, F., Trypanosomes et trypanosomiases, 2d edition, Paris, 1912, 241.

[2] Jürgens, *Arch. f. Hyg.*, 1902, xlii, 265.

[3] Delanoë, P., *Compt. rend. Soc. de biol.*, 1911, lxx, 649.

[4] Roudsky, D., *Compt. rend. Soc. de biol.*, 1910, lxviii, 421, 458; 1911, lxx, 741.

[5] Wendelstadt, H., and Fellmer, T., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 422; 1910, v, 337.

raising the virulence of *Trypanosoma lewisi* by passage through cold-blooded animals.

These facts suffice to show the existence of pathogenic strains of *Trypanosoma lewisi* and indicate that possibly all strains possess potential pathogenic properties. Unfortunately, the known facts regarding its pathogenicity are too meagre to warrant any generalizations. Since the natural host of this organism makes it peculiarly suited to laboratory study, further work upon this subject seems highly desirable, as a clearer comprehension of the conditions governing its pathogenicity would probably aid materially in advancing our knowledge of the more important group of organisms, —the pathogenic trypanosomes.

Recently, we have had under observation a strain of *Trypanosoma lewisi* that, for a short time, showed an unusual increase in its virulence, and an account of the action of this organism is offered as a contribution to the study of its pathogenicity.

A complete genealogy of this strain of *Trypanosoma lewisi* is not available. The organism was isolated from a natural infection in a wild rat and has been carried for several years in white rats with no unusual manifestations of virulence. In October, 1913, for three successive generations, fatal infections resulted from the intraperitoneal injection of one to two drops of tail blood, diluted with one cubic centimeter of a 1 per cent. sodium citrate solution, death taking place eight to eleven days after inoculation. Five rats out of the six inoculated succumbed to this infection. At first it appeared improbable that *Trypanosoma lewisi* was entirely responsible for such an unusually high mortality, but as careful investigation, including cultures from the peritoneal cavity and heart's blood, failed to reveal any other cause, the following test of virulence was applied.

Experiment 1.—Nov. 28, 1913. A large rat infected with strain I of *Trypanosoma lewisi* was bled to death on the twelfth day of infection and the blood defibrinated. At this time there was a heavy blood infection with a few multiplication forms still present. The rat was weak and showed a marked anemia and loss of weight. With aseptic precautions, ten normal rats were injected intraperitoneally with 0.2 c.c. of blood diluted with 0.8 c.c. of an 0.85 per cent. salt solution. Five of the rats weighed between 100 and 170 gm., and the other five between 40 and 70 gm. As a control, four normal rats (two large and two small) of the same lot were kept under observation and two large immune rats were injected intraperitoneally with 0.5 c.c. of blood in 1 c.c. of salt solution.

Nine of the normal rats inoculated showed trypanosomes in their blood within twenty-four hours. The tenth rat, a large one, showed a very few organisms at forty-eight hours, but the infection was transient, disappearing completely within five days without any demonstrable multiplication having taken place; this rat was probably an immune. Another large rat died in forty-eight hours and the autopsy showed a marked bronchiectasis. If we exclude these two rats from the list of normal ones, the eight remaining rats showed a rapid increase in trypanosomes in the blood with definite multiplication on the third day, persisting until the fatal termination of the infection. The rats became torpid and weak, with marked anemia and dyspnea, and slight loss of weight. A few rats showed bloody nasal and lachrymal discharges, and all of them developed a diarrhea with greater or less abdominal distension. Two of the large rats died on the sixth day, and the third died on the seventh day, while all five of the small rats died on the ninth day after inoculation.

Autopsies on these rats showed an acute enteritis, marked splenic enlargement and hyperplasia of the bone marrow, and a few foci of necrosis in the liver. Several rats showed a moderate pulmonary congestion but no bronchiectasis or pneumonia. Cultures made immediately after death from the peritoneal cavity and from the heart's blood of three rats remained sterile. No cultures were taken from the other rats.

The normal and immune controls remained unaffected for one month, when observations were discontinued.

The existence of nasal and lachrymal discharges and diarrhea in these rats would naturally incline one to suspect some condition complicating the trypanosomiasis. In an attempt to clear up the relation of these conditions to the trypanosomal infection, a number of observations have been made on normal and immune rats inoculated with different strains of *Trypanosoma lewisi.* These observations show that the above conditions may recur frequently in a given series of inoculations while they do not appear in control immunes inoculated from the same source, in uninoculated normal rats from the same lot, or in rats inoculated with another strain of *Trypanosoma lewisi.* Likewise, normal and immune rats kept in

contact with infected rats showing these conditions, with a single exception, have not developed these symptoms except as the normal rats became infected with trypanosomes by natural means. The fact that the same symptoms occur in the course of other trypanosomal infections in the rat indicates that they may be a part of the morbid manifestations of trypanosomiasis. Whether these conditions are the result of an uncomplicated trypanosomal infection or the result of a latent infection whose development is favored by the trypanosomiasis is not clear. In either case, however, it is evident that it is the trypanosome that is directly or indirectly responsible for the condition.

The series of fatal infections in the stock transfers of this organism was interrupted by inoculating two young rats weighing seventy grams, with one drop of tail blood, taken on the tenth day of infection, from the rat killed for the above experiment. One of the rats was extremely ill for about twenty days but finally recovered. The infection in the other rat was much less severe. On the next transfer from the first of these rats, one large and one small rat were inoculated and the resulting infections were still further decreased in severity.

The small rat of this series was then sacrificed for a second test of the virulence of this strain of *Trypanosoma lewisi*, in this instance contrasted with another strain, recently isolated from a natural infection in a white rat.

Experiment 2.—Dec. 17, 1913. A young rat infected with strain I of *Trypanosoma lewisi* and another young rat infected with strain V were bled from the heart on the eighth day of infection and the blood was collected and defibrinated under aseptic conditions. The blood of the two rats showed about an equal number of trypanosomes with many multiplication forms. From each of these rats, five rats weighing 80 to 90 gm. were inoculated intraperitoneally with 0.2 c.c. of blood diluted with 0.8 c.c. of salt solution.

The five rats infected with strain I all showed an incubation period of less than twenty-four hours. Three of the five rats developed a severe infection with weakness, a high grade anemia, and loss of weight. The other two showed only a moderate infection. None of the rats died and trypanosomes were present in the blood of four on the thirtieth day, when observations were discontinued. Of the five rats infected with strain V, the incubation period of four was

less than twenty-four hours, of the fifth between thirty-six and forty-eight hours. Only one of these rats showed any appreciable disturbance from the infection. This rat showed an extreme blood infection, with weakness, torpor, anemia, and loss of weight, persisting for about three weeks. Trypanosomes disappeared from the blood of two of these rats within twenty days and from a third by the twenty-sixth day.

Although none of the ten rats died from the infection, the difference in the severity of the infection produced by the two strains of *Trypanosoma lewisi* was sufficiently well marked to show that strain I was decidedly more virulent than strain V.

This series of observations lends support to the idea that there are strains of *Trypanosoma lewisi* that differ fundamentally as to their pathogenicity, but what is of even greater importance is that any particular strain is subject to marked fluctuations of virulence. From these facts it would seem important to determine the conditions that give rise to such alterations of virulence as have been described, the extent to which these variations of virulence more or less permanently modify the characteristics of the strain, and, finally, whether there are differences in morphology corresponding with variations in virulence or with the differences in pathogenicity exhibited by different strains of *Trypanosoma lewisi.* These subjects are now under investigation.

CONCLUSIONS.

1. Some strains of *Trypanosoma lewisi* may, at times, produce rapidly fatal infections in a large percentage of the rats infected.

2. In such strains of *Trypanosoma lewisi,* a sufficient degree of pathogenicity may persist to warrant the designation of these strains as pathogenic.

3. The pathogenicity of a given strain of *Trypanosoma lewisi* is not constant, but is subject to marked and even sudden variations.

PENETRATION OF THE VIRUS OF POLIOMYELITIS FROM THE BLOOD INTO THE CEREBRO-SPINAL FLUID.*

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An active poliomyelitic virus readily causes infection in monkeys when introduced into the brain, subarachnoid spaces, or peripheral nerves. When, however, the virus is injected into the circulation, far greater quantities are required to produce infection and the onset of the usual symptoms and the paralysis are delayed.[1] Were the virus withdrawn from the blood directly by the tissues of the central nervous system neither the greater dose nor the longer incubation should be necessary. It appears that the extranervous organs retain but little of the virus; hence we may suppose that the route by which the virus contained within the blood reaches the central nervous system is an indirect one.

It is now generally conceded that the poliomyelitic virus enters the human body by way of the upper respiratory passages, and in particular through the nasopharyngeal mucous membrane. Once within this membrane the virus may pass through the lymphatic channels surrounding the filaments of the olfactory nerve to the leptomeninges where it reaches the cerebrospinal fluid, or it may first enter the blood and be conducted to the central nervous organs by the general circulation. Flexner and Clark[2] have shown experimentally that when the virus is introduced into the upper nasal mucosa in monkeys its propagation can be followed from the olfactory lobes of the brain to the medulla oblongata and spinal cord. Had the distribution of the virus taken place by way of the general circulation the several parts of the nervous organs should have been

* Received for publication, February 26, 1914.

[1] Clark, P. F., Fraser, F. R., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 223.

[2] Flexner, S., and Clark, P. F., *Proc. Soc. Exper. Biol. and Med.*, 1912–13, x, 1.

rendered infectious almost simultaneously. The virus of poliomyelitis has hitherto been regarded as strongly neurotropic; but it does not follow from the fact of this neurotropic affinity that the nervous tissues can under all circumstances remove the virus from the blood.

Another possibility exists, namely, that the virus introduced into the blood finds its way not directly to the nervous organs, but indirectly by way of the cerebrospinal fluid. When, therefore, a quantity introduced into the blood is insufficient to cause infection, although a much smaller quantity produces infection when introduced into the brain, the reason may be that the intact choroid plexus prevents the virus from reaching the cerebrospinal liquid. It is well known that this anatomical barrier excludes from the cerebrospinal fluid many substances contained within the blood, that the barrier is not absolute but is capable of being broken down, and that the most frequent source of injury is the pathogenic action of infectious microörganisms.

Hence we may consider that when insufficient quantities of the poliomyelitic virus are introduced into the blood they do not set up poliomyelitis because they fail to injure the choroid plexus; and when poliomyelitis is set up by larger quantities the plexus has been penetrated.

The virus has thus far not been detected by inoculation experiments in the cerebrospinal fluid obtained from human cases of poliomyelitis; and, indeed, when the virus is injected into the subarachnoid spaces in monkeys it remains within the fluid for a limited time and can no longer be detected there at the period of the onset of paralysis.[3] The conditions within the fluid are obviously unfavorable for the propagation of the virus; but the fluid constitutes the most immediate route for the passage of the virus to the interior of the nervous organs in which it multiplies and becomes fixed. The cerebrospinal fluid therefore acts merely as a medium for transporting the virus to the nervous tissues. Hence, should the passage of the virus contained within the blood proceed to the nervous organs by way of the cerebrospinal fluid it should be possible to detect it in transit. From the many failures to discover the virus in

[3] Clark, Fraser, and Amoss, *loc. cit.*

the early paralytic stages of poliomyelitis in man and the monkey it might appear that the probability of detecting the virus at all might be small. But it would now appear that when a large dose of the virus has been injected into the blood and a sufficient period allowed to elapse in order that the virus may act upon and injure the choroid plexus, it is possible to detect the presence of the virus in the cerebrospinal fluid by inoculation.

EXPERIMENTAL.

A passage strain of highly active K virus was employed for the intravenous inoculation. A Berkefeld filtrate of a 5 per cent. suspension caused paralysis and death when inoculated intracerebrally in doses of 0.2 to 0.3 of a cubic centimeter. For the purpose of intravenous inoculation the 5 per cent. suspension was merely centrifugalized and the clear supernatant fluid pipetted off. It was not filtered.

Experiment A. Macacus rhesus 1.—Jan. 30. 250 c.c. of the supernatant fluid were injected into a superficial vein of the leg. No effect was produced by the injection. No symptoms appeared until Feb. 14, when slight excitability was noted. Feb. 16. Weakness of the leg. Feb. 17. Excitability increased and arms paralyzed. Feb. 18. Legs and back paralyzed. Feb. 19. Died.

Lumbar puncture was performed as follows: on Feb. 1 (48 hours after the injection) 0.6 c.c. of cerebrospinal fluid was removed; on Feb. 2 (72 hours after the injection) 0.5 c.c. of fluid removed; on Feb. 3 (96 hours after the injection) 0.9 c.c. of fluid removed; and on Feb. 18 (19 days after the injection) 2.9 c.c. of fluid removed. The several samples of cerebrospinal fluid were free from blood. On Feb. 18 a sample of blood was also taken and defibrinated.

The specimens of cerebrospinal fluid and blood were injected intracerebrally into monkeys as follows:

Macacus rhesus 2.—Feb. 1. 0.6 c.c. of cerebrospinal fluid withdrawn from monkey 1 48 hours after an intravenous injection of K virus was inoculated intracerebrally. No symptoms were produced; the monkey remained well.

Macacus rhesus 3.—Feb. 2. 0.5 c.c. of cerebrospinal fluid withdrawn from monkey 1 72 hours after an intravenous injection of K virus was inoculated intracerebrally. Feb. 14. Monkey somewhat excitable, movements slow. The condition remained stationary until Feb. 25, when the excitability was more marked. No weakness of muscles was detected. Lumbar puncture yielded a clear fluid devoid of globulin and containing forty white corpuscles per c.mm. Feb. 26. Condition not so good; ataxia; slight weakness of both arms. Mar. 10. Condition stationary.

Macacus rhesus 4.—Feb. 3. 0.9 c.c. of cerebrospinal fluid withdrawn from

monkey 1 96 hours after an intravenous injection of K virus was inoculated intracerebrally. Feb. 7. Excitability. The condition did not change materially until Feb. 17 when excitability was greater. The condition again became stationary until Feb. 26, when a general paralysis of the muscles was present. Animal etherized. Lesions of poliomyelitis present.

Macacus rhesus 5.—Feb. 18. 2.9 c.c. of cerebrospinal fluid withdrawn from monkey 1 19 days after an intravenous injection of K virus and the 1st day of paralysis were inoculated intracerebrally. Feb. 26, A. M. Excitable; tremor; ataxic. P. M. Left arm flaccid. Feb. 27. Animal prostrate; all members were paralyzed except the tail and a few neck muscles. Etherized. Lesions of poliomyelitis present.

Macacus rhesus 6.—Feb. 18. 5 c.c. of defibrinated blood withdrawn from monkey 1 19 days after an intravenous injection of K virus and the 1st day of paralysis were inoculated intracerebrally. No symptoms developed; the animal remained well.

The experiments bring out several facts. (1) Even a very large dose of an active poliomyelitic virus when injected into the blood produces a much delayed infection. If we consider the certain effective dose of the specimen of K virus employed when injected intracerebrally at 0.2 of a cubic centimeter, then 1,250 doses were introduced into the blood of monkey 1. The average incubation period after an intracerebral inoculation is about 6 days;[4] in monkey 1 the period was 17 days. (2) The cerebrospinal fluid removed 48 and 72 hours, respectively, after the intravenous injection failed, when inoculated intracerebrally, to communicate definite poliomyelitis to rhesus monkeys; while the fluid removed 96 hours (monkey 4) and 19 days (monkey 5) after the intravenous injection caused typical poliomyelitis in monkeys of this species. In monkey 4 the incubation period was indefinite and in monkey 5 it was 8 days. (3) By the 19th day following the intravenous injection of the large dose of the virus and at the onset of the paralysis the virus had disappeared from the blood while it was still detectable in the cerebrospinal fluid by inoculation.

DISCUSSION.

The experiments emphasize in the first place the relatively great difficulty of infecting monkeys with the virus of poliomyelitis by introducing it directly into the blood. At first sight it may appear that this statement is in conflict with the effects of subcutaneous or

[4] Clark, Fraser, and Amoss, *loc. cit.*

even of intraperitoneal inoculations. It is, however, not improbable that in all external modes of inoculation practiced, except the intravenous mode, the virus actually penetrates to the central nervous organs by way of the nerves. In any event the difficulties in the way of accomplishing infection through the general blood provide another argument against the notion that the virus of epidemic poliomyelitis is communicated to man by the bite of infected blood-sucking insects.

On the other hand, the experiments afford valuable support to the hypothesis that infection of the nervous organs in man occurs through the mediation of the cerebrospinal fluid. The virus readily traverses the nasal mucous membrane to reach this fluid, which is capable of carrying the virus to the interstices of the nervous tissues. Apparently the virus enters the intimate structures of the nervous tissues not directly from the blood but indirectly after being passed from the blood to the cerebrospinal fluid. To accomplish this transfer time is required since the barrier of the choroid plexus must first be overcome. At the expiration of 48 hours following the intravenous inoculation the barrier appears still to be intact; at the expiration of 72 hours the passage of the virus seems to have begun, since infection of mild type followed the inoculation of the cerebrospinal fluid removed at this period. At the expiration of 96 hours it appears that the barrier had broken down; and it also appears that under the pathological conditions created the virus, in quantity sufficing to cause infection, still persisted in this fluid as late as the 19th day, although no longer detectable in the blood by the inoculation test. In no other instance has the virus been found in the cerebrospinal fluid at the period of the onset of paralysis.

When we consider the minute size of the microörganism constituting the virus of poliomyelitis we may well wonder at the failure to penetrate the capillaries to gain access to the interstices of the central nervous organs. The case is not wholly unique. von Behring discovered that the hen, which is insusceptible to the action of tetanus toxin injected into the blood, is subject to its effects when introduced into the cerebrospinal fluid. Lesions of the leptomeninges of an interstitial character are implicated in the develop-

ment of the specific lesions of poliomyelitis. They also suffice to produce at times in man and the monkey a poliomyelitic affection of the meninges in which the central nervous organs proper do not share specifically. Hence the meninges and the cerebrospinal fluid play a highly important and even a determining part in the pathogenesis of epidemic poliomyelitis.

CONCLUSIONS.

The virus of poliomyelitis introduced into the blood may pass indirectly by way of the cerebrospinal fluid to the interstices of the central nervous organs.

To reach the cerebrospinal fluid the virus must first penetrate the barrier of the choroid plexus, which operation requires time. By the inoculation test, no virus was detected in the fluid at the expiration of 48 hours, only small amounts at the expiration of 72 hours, while at the expiration of 96 hours the virus had passed more freely. The virus was still detectable in the fluid at the onset of paralysis 19 days after the intravenous injection.

Pathological conditions of the leptomeninges and the cerebrospinal fluid play an important part in the pathogenesis of epidemic poliomyelitis.